

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problems Mailbox.**

STIC-ILL

9/21/04

From: Steadman, David (AU1652)
Sent: Wednesday, February 13, 2002 1:57 PM
To: STIC-ILL
Subject: Literature/ILL request

Art Unit: 1652
Office: 10D-04
Mailbox: 10C-01 M3
Case Serial #:09/720,096

Please provide the following reference:

1) J Biol Chem 1990 Nov 5;265(31):18776-9
Saturation site-directed mutagenesis of thymidylate synthase.
Climie S, Ruiz-Perez L, Gonzalez-Pacanowska D, Prapunwattana P, Cho SW, Stroud R, Santi DV.

2) Microbiology 1994 Oct;140 (Pt 10):2601-10
A Lactococcus lactis gene encodes a membrane protein with putative ATPase activity that is homologous to the essential Escherichia coli ftsH gene product.
Nilsson D, Lauridsen AA, Tomoyasu T, Ogura T

Thank you very much,
David J. Steadman
Art Unit 1652
CM1, 10D-04
308-3934

A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli* *ftsH* gene product

Dan Nilsson,¹ Anette A. Lauridsen,¹ Toshifumi Tomoyasu² and Teru Ogura²

Author for correspondence: Dan Nilsson. Tel: +45 45 767 676. Fax: +45 45 765 455.
e-mail: dnai@biobase.aau.dk

¹ Department of Genetics, Chr. Hansen's Laboratorium Danmark A/S, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark

² Department of Molecular Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kumamoto 862, Japan

A gene, encoding a protein homologous to an essential *Escherichia coli* protein, FtsH, was identified adjacent to the *hpt* gene and the *trnA* operon in the Gram-positive bacterium *Lactococcus lactis*. The deduced amino acid sequence of the gene product showed full-length similarity to FtsH of *E. coli*, Yme1p of *Saccharomyces cerevisiae* and a conserved region found in a new family of putative ATPases. In-frame fusions of *L. lactis* *ftsH* and *phoA1* in *E. coli*, and immunodetection of the *L. lactis* FtsH protein in cell fractions using anti-*E. coli* FtsH serum showed that *L. lactis* *ftsH* was expressed and encodes a membrane protein. When contained on a high copy number plasmid, the *L. lactis* *ftsH* gene complemented the lethality of a Δ *ftsH3::kan* mutation in *E. coli* at 37 °C and below, indicating that the *L. lactis* *ftsH* gene can functionally replace the *E. coli* *ftsH* gene to some extent. The resulting *E. coli* strain showed temperature sensitivity and salt sensitivity. A *L. lactis* mutant with an insertion into *ftsH* was salt-, heat- and cold-sensitive. These results suggest that FtsH is somehow involved in stress responses. Southern hybridization analysis indicated that genes homologous to *ftsH* of *L. lactis* were also present in *Bacillus subtilis*, and several *Lactobacillus* and *Leuconostoc* species, suggesting high conservation of *ftsH* in bacterial species.

Keywords: *Lactococcus lactis*, *ftsH*, (*tma*), membrane protein, AAA-protein family

INTRODUCTION

The facultative anaerobic Gram-positive bacterium *Lactococcus lactis* subsp. *lactis* is widely used as an industrial organism for homolactic fermentation. *L. lactis* has become a model organism for fundamental genetic research on plasmids and plasmid-encoded functions involved in the production of fermented food products. However, the genetic characterization of chromosomally encoded functions in *L. lactis* has only recently been started. We initiated a genetic approach to study purine metabolism in *L. lactis* and have characterized the *hpt* gene encoding hypoxanthine guanine phosphoribosyl-transferase, an enzyme involved in the salvage of purine bases to the corresponding nucleotides (Nilsson & Lauridsen, 1992). During this work we detected part of an

open reading frame adjacent to *hpt*, transcribed in the same direction, encoding a putative membrane protein.

In this report we describe the cloning and characterization of the *L. lactis* gene, previously named *tma*, adjacent to *hpt* and the tRNA operon *trnA* (Nilsson & Johansen, 1994). We show that the gene is homologous to the *Escherichia coli* *ftsH* gene, and that this gene is conserved in several Gram-positive bacteria, including lactic acid bacteria. We suggest that the gene be designated *ftsH*.

The *ftsH* genes of *E. coli* and *L. lactis* encode members of a new family of ATPases, the AAA-protein family (Kunau *et al.*, 1993). The family includes both eukaryotic and prokaryotic members which have been reported to be involved in diverse cellular activities, e.g. cell cycle control, protein secretion, peroxisome biogenesis and proteolysis.

E. coli *ftsH* mutants show pleiotropic phenotypes: defects in cell growth and cell viability (Ogura *et al.*, 1991;

The GenBank accession number for the nucleotide sequence reported in this paper is X69123.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant features	Source or reference
<i>Escherichia coli</i>		
XL1-Blue	<i>end.A1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1</i> $\Delta(lac)$ [F' <i>proAB lacI^qZ</i> Δ M15 Tn10]	Stratagene, La Jolla
DH5 α	ϕ 80 <i>lacZ</i> Δ M15 $\Delta(lacZYA-argF)$ U169 <i>recA1</i> <i>end.A1 hsdR17 supE44 thi-1 gyrA96 relA1</i>	Hanahan (1983)
SØ609	<i>ara thi rpsL</i> $\Delta(pro-gpt-lac)$ <i>hpt deoD purD</i>	Jochimsen <i>et al.</i> (1975)
CC118	<i>araD139</i> $\Delta(ara-leu)$ 7697 $\Delta lacX74$ <i>phoA</i> Δ 20 <i>galE</i> <i>galK thi rpsE rpoB argEam recA1</i>	Manoil & Beckwith (1986)
AR423	<i>met gal supE hsdR sfiC</i> $\Delta(srl-recA)$ 306::Tn10 $\Delta ftsH3::kan$ [pAR171 <i>ftsH rep^{ts}</i> Cm ^R]	Akiyama <i>et al.</i> (1994a)
AR3120	<i>met gal supE hsdR sfiC</i> $\Delta(srl-recA)$ 306::Tn10 $\Delta ftsH3::kan$ [pLN32 <i>L. lactis ftsH</i>]	This study
<i>Lactococcus lactis</i>		
CHCC285	Wild-type	Nilsson & Lauridsen (1992)
MG1363	Plasmid-free	Gasson (1983)
DN4302	MG1363 <i>ftsH::pLN43</i>	This study
Plasmids		
pBluescript KS+	Cloning vector (Ap ^R)	Stratagene
pBluescript SK-	Cloning vector (Ap ^R)	Stratagene
pVS2	Ery ^R Cm ^R	von Wright (1987)
pV2	pBluescript KS+ with a 1.3 kb <i>ClaI</i> - <i>HpaII</i> fragment (Ery ^R) from pVS2	Svend E. W. Hansen (personal communication)
pAR171	<i>ftsH rep^{ts}</i> Cm ^R	Akiyama <i>et al.</i> (1994a)
pLN2	pBluescript SK- with a 1.9 kb <i>EcoRI</i> - <i>MboI</i> fragment (<i>ftsH'</i>) from CHCC285	Nilsson & Lauridsen (1992)
pLN3	pBluescript SK- with a 1.3 kb <i>HindIII</i> - <i>MboI</i> fragment (<i>ftsH'</i>) from CHCC285	Nilsson & Lauridsen (1992)
pLN22-27	pLN2 <i>ftsH::Tn5phoA1</i>	This study
pLN30	pBluescript KS+ with a 7.3 kb <i>SpeI</i> fragment (<i>hpt ftsH</i>) from CHCC285	This study
pLN32	pBluescript KS+ with a 3.2 kb <i>EcoRI</i> fragment (<i>ftsH</i>) from pLN30	This study
pLN39	pBluescript KS+ with a 1.0 kb <i>HindIII</i> - <i>EcoRI</i> fragment (<i>ftsH</i>) from pLN32	This study
pLN43	pV2 with a 1.3 kb <i>EcoRV</i> fragment (<i>ftsH'</i>) from pLN32	This study

Tomoyasu *et al.*, 1993a), defects in protein assembly into and through the membrane (Akiyama *et al.*, 1994a, b; Tomoyasu *et al.*, 1993a) and a defect in a proteolytic pathway (Herman *et al.*, 1993). A *Bacillus subtilis ftsH* mutant shows salt sensitivity (Geisler & Schumann, 1993). However, it has not satisfactorily been demonstrated how *ftsH* mutations cause these diverse defects, and what the function of FtsH is. In this respect, it is interesting to know the structural similarity and functional relationship of FtsH in Gram-negative and Gram-positive bacteria. Comparative studies on the FtsH proteins in Gram-positive and Gram-negative bacteria

will provide better understanding of the function of this highly conserved putative membrane ATPase.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used are listed in Table 1. *L. lactis* was grown in M17 medium (Oxoid), containing 0.5% glucose, routinely at 30 °C. *E. coli* and *B. subtilis* were grown in L-broth (Miller, 1972) at 37 °C unless otherwise stated. The phosphate-buffered minimal medium of Clark & Maaløe (1967) was used for selection of SØ609 Hpt⁺ as described previously (Nilsson & Lauridsen, 1992). For growth of plasmid-containing cells, the

media contained appropriate antibiotics: *E. coli*, ampicillin (50 mg l⁻¹), kanamycin (300 or 30 mg l⁻¹), chloramphenicol (10 mg l⁻¹); *L. lactis*, erythromycin (1 mg l⁻¹). For identification of blue PhoA⁺ colonies on agar plates, 40 mg XP1⁻¹ (5-bromo-4-chloro-3-indolyl phosphate, Sigma) was added.

DNA manipulations and sequencing. *L. lactis* chromosomal DNA was isolated according to Johansen & Kibbenich (1992). *E. coli* and *B. subtilis* chromosomal DNA was isolated as described by Silhavy *et al.* (1984) and *E. coli* plasmids as described by Birnboim & Doly (1979). The use of restriction enzymes, T4 DNA ligase and calf intestine alkaline phosphatase were as recommended by the suppliers (Boehringer Mannheim, Promega, Stratagene). Plasmid transformation of *E. coli* and *L. lactis* was performed according to Mandel & Higa (1970) and Holo & Nes (1989), respectively. The nucleotide sequence of both strands of DNA was determined by the dideoxy method (Sanger *et al.*, 1977). Universal primers from Stratagene or customized primers were used as sequencing primers. All nucleotide sequence data were processed and the deduced amino acid sequences compared using the GCG software package, version 7.0 (Devereux *et al.*, 1984) and EMBL nucleotide sequence database release 37.0.

Cloning of ftsH. A *L. lactis* strain CHCC285 chromosomal DNA library, containing 1–10 kb *SpeI* restriction fragments in the bacteriophage vector λ Zap II (Stratagene) was used. By selecting for Hpt⁺ colonies of *E. coli* strain S0609 (Nilsson & Lauridsen, 1992), we isolated clone λ LN2 containing a 7.3 kb and a 1.3 kb *SpeI* fragment. The 7.3 kb fragment, containing the *hpt* and *ftsH* genes, was subcloned in the plasmid vector pBluescript KS+ (Stratagene) resulting in pLN30 (Table 1).

Construction of pLN2::Tn5phoA1. Plasmid pLN2 (Table 1), which contains the first 441 codons of the *ftsH* gene, was used to transform *E. coli* strain CC118. Following infection of CC118(pLN2) with *irex*::Tn5phoA1 b221 c1857 Pam3 (Gutiérrez *et al.*, 1987), blue colonies on LB XP agar plates (PhoA⁺) that were Kan^R and Ap^R, were selected and pooled. Plasmids were isolated and used to transform CC118 to PhoA⁺, Ap^R and Kan^R. Six independent pLN2::Tn5phoA1 isolates were obtained.

Identification of L. lactis FtsH in E. coli. Various *E. coli* extracts from strains containing plasmids were used for SDS-PAGE and subsequently blotted semi-dry to nitrocellulose as described by Ipsen & Larsen (1988). The nitrocellulose was subsequently immunostained (Larsen *et al.*, 1992) using the anti-*E. coli* FtsH serum described previously (Tomoyasu *et al.*, 1993b).

Cell-fractionation of L. lactis. Cells were grown in 400 ml of M17 medium to an OD₆₀₀ of 0.9–1.0, harvested and resuspended in 5 ml 30 mM sodium phosphate (pH 6.8) containing 0.4 M sucrose and 1 mg lysozyme ml⁻¹. After incubation at 37 °C for 1 h, the cells were harvested, washed twice with 30 mM sodium phosphate containing 0.4 M sucrose, resuspended in 5 ml 10 mM sodium phosphate (pH 6.8) and sonicated. After ultracentrifugation of the cell extract (100 000 g, 1 h at 4 °C), the supernatant (cytoplasmic) fraction was collected. The pellet (membrane) fraction was resuspended in 10 mM sodium phosphate containing 1 M NaCl and ultracentrifuged as above. The supernatant and pellet were collected separately. The supernatant was desalted by gel filtration (Sephadex G-25M, Pharmacia).

The collected fractions were used for SDS-PAGE, blotted to nitrocellulose and immunostained with anti-*E. coli* FtsH serum as described above.

Southern hybridization analysis. A 1.0 kb *HindIII*–*KpnI* (the *KpnI* site originates from the polylinker of pBluescript SK–)

restriction enzyme fragment of plasmid pLN3 (Table 1) contains an internal part of the *L. lactis* *ftsH* coding region. This fragment was used as probe in Southern hybridization analysis with *ScaI*-digested chromosomal DNA from various bacterial strains, using the ECL Gene Detection System, version 2 (Amersham) as follows. In the hybridization buffer NaCl was added to a final concentration of 1.0 M; primary washes were in 2 × SSC, 1 M urea, at 25 °C. All other procedures including probe labelling, DNA transfer and signal detection were as described by Amersham. The molecular mass markers were obtained from Gibco BRL.

To verify the integration of pLN43 (Table 1) into the *ftsH* gene of *L. lactis* strain DN4302 *ftsH*::pLN43, a 1.3 kb *EcoRV* internal fragment of *ftsH* was used as probe in Southern hybridization analysis with *EcoRI*-digested chromosomal DNA from DN4302 and *L. lactis* strain MG1363 wild-type. Standard conditions as recommended by Amersham were used.

RESULTS

Nucleotide sequence of L. lactis ftsH

Adjacent to the *hpt* gene and *trnA* operon of *L. lactis*, a gene (*ftsH'*) was found (Nilsson & Lauridsen, 1992; Nilsson & Johansen, 1994). The complete *ftsH* gene was cloned and the nucleotide sequence was determined. The sequence of the last 24 codons of *hpt* and the entire *ftsH* gene is shown in Fig. 1. A possible ribosome binding site (Ludwig *et al.*, 1985; Shine & Dalgarno, 1974) and a putative promoter (Koivula *et al.*, 1991) are located upstream of *ftsH* (Fig. 1). The putative promoter contains the sequence 5' ATATG 3' in the –16 region (consensus 5' RTRTG 3' where R = purine), which is found in strong *B. subtilis* promoters (Henkin *et al.*, 1988; Moran *et al.*, 1982). A potential stem-loop structure followed by five Ts (nucleotides 82–101) was located between the *hpt* and *ftsH* coding regions. The translation stop codon of the *ftsH* gene was located in the loop of a potential transcription terminator structure (nucleotides 2352–2391).

Analysis of the deduced amino acid sequence of the *ftsH* gene revealed a purine nucleotide binding site motif (residues 233–240 and 288–293) (Walker *et al.*, 1982). Two putative transmembrane sequences can be predicted (residues 1–29 and 136–158) (Kyte & Doolittle, 1982). The transmembrane sequence (residues 136–158) and the region containing the purine nucleotide binding site are separated by a very glycine rich sequence (residues 159–170, GGMGARGGGGGG).

Comparison of L. lactis FtsH with other proteins

Database searches for genes encoding similar amino acid sequences revealed that the deduced amino acid sequence of *L. lactis* *ftsH* was 47% identical to that of *E. coli* *ftsH* (Tomoyasu *et al.*, 1993a) and 36% identical to that of *Saccharomyces cerevisiae* *Yme1* (Thorsness *et al.*, 1993). The deduced *L. lactis* FtsH amino acid sequence contains a region of 200 amino acid residues that reveals similarity to conserved domains in the AAA-protein family of putative ATPases (Erdmann *et al.*, 1991; Kunau *et al.*, 1993). In this region the *L. lactis* FtsH amino acid

	TTGGACTACGAAGAAACTATCGTAATCTTCCATATGTCGGAGTTTTAAACCAGAAGTTATAACAAATAATTCGTTGATTTAGTCATCTAAAGTTTT	100
	L D Y E E N Y R N L P Y V G V L K P E V Y N K *	
	-35 -16 -10	
	TCTCAGACTAAAGTATGAAAAATCTCTGACCAATACTTACTATTAGCATTCCAAAAGGAAATATGCTATAACTAGGTATAGCTGTTATTGACTAGCCTG	200
	RBS Tma	
1	TATCCTTTATTACAGTGATACTAAGAAGTAAATCTTGGCTTTTCTAATAATAAATAACAAAAGATAAGGAAATATGAATAACAACAACCAACAA	300
	M N N N K Q P K	
9	ACAAGGAAATTTTGAAAAATATCTTGATGTGGGTAATCTTGGCTATTGTTGTTGTTGTCGGTTTCAATTTCTTCTCAGTAGTAATCAATCAAGCGTG	400
	Q G N F V K N I L M W V I L A I V V V V G F N F F S S N Q S S V	
42	GATAAAATTAGCTATTACAATTGATGACGAACTTGACGGTAACAAGATTGAAACGTCACAATGCAACCTTCTGATAGCTTAATTACTGTAACAGGTG	500
	/phoA(24) /phoA(26)	
	/phoA(25)/phoA(27)	
76	AATATAAAGAACCTGTAAAGTAAAGGAACAAATAATTTCCCACTTTTAGGCAATCTAGTAGTGAAGTTAAAACTTCCAAGCTTATATTATTCCAAC	600
	Y K E P V K V K G T N N F P L L G N S S S E V K N F Q A Y I I P T	
	EcoRV /phoA(22)/phoA(23)	
109	TGACAGTGTGTCAAGGATATCCAAATGCAGCTAAAAGTAATGATGTAAAACCTAGTGTGTTCAAGCTTCATCAAGTGGTATGTGGGTTCAAATTTCTC	700
	D S V V K D I Q N A A K S N D V K L S V V Q A S S S S G M W V Q I L	
142	TCATACATCATTTCAATGCTTCTATTGTTGGTATCTTCTGGCTCATGATGGGCGGAATGGGCGCTCGTGGCGGAGGCGCGGTGGAAATCCGATGTCCT	800
	S Y I I P M L L F V G I F W L M M G G M G A R G G G G G G N P M S F	
176	TCGGTAAATCTCGTGCTAAACAACAAGATGGTAAACATCTAAAGTTCGTTTTGCTGACGTTGCCGGTCTGAAGAGGAAAAACAAGAGCTTGTAGAAGT	900
	G K S R A K Q Q D G K T S K V R F A D V A G S E E E K Q E L V E V	
209	TGTTGATTTCTTAAAAATCCGAAAAATATCATGATTAGGAGCTCGTATCCAGCAGGTGTTCTTCTGAAGGCCCTCCAGGTACAGGTAAACATTG	1000
	V D F L K N P K K Y H D L G A R I P A G V L L E G P P G T G K T L	
242	CTTGCTAAGGCTGTTGCCGGTGAAGCAGGAGTTCCTTTCTATAGTATCTCAGGTTCTGATTTGTTGAAATGTTTGTGGTGTGGTGCCTCACGTGTCC	1100
	L A K A V A G E A G V P F Y S I S G S D F V E M F V G V G A S R V R	
276	GTGACTTATTTGAAATGCTAAGAAAAGTGCACCATCAATTATCTTTATGATGAAATGATGCTGTTGGTGGTCAACGTTGGTGCAGGTCTTGGTGGTGG	1200
	D L F E N A K K T A P S I I F I D E I D A V G R Q R G A G L G G G	
309	TAACGATGAACGTGAACAAACCTTAACCAATTGCTCGTTGAAATGGATGGTTTCCAAGATGATGGCAACTCAGTAATCGTTATTGCTGCAACTAACCGT	1300
	N D E R E Q T L N Q L L V E M D G F Q D D G N S V I V I A A T N R	
342	TCAGATGTGCTTGACCCAGCGCTTTTACGTCCAGGTGCTTTTACCCTGAAAGTCTTGGTGGAGCTCCAGATGTTAAAGTCTGTAAGCCGTTCTTAAAG	1400
	S D V L D P A L L R P G R F D R K V L V G A P D V K G R E A V L K V	
376	TTCATGCTAAAAACAACCTTTAGCAAGTGATGTTGATTTGCACAAATGTTGCTACACAACTCCAGGCTATGTCGGAGCTGATTTGAAAAATGTTTGA	1500
	H A K N K P L A S D V D L H N V A T Q T P G Y V G A D L E N V L N	
409	TGAAGCTGCACTGTTGCTGCACGTCAAAATAAAAAGAAATCAATGCTGCTGACATTGATGAAGGAATGGACCGTCAATGGCTGGTCCAGCTAAGAAA	1600
	E A A L V A A R Q N K K E I N A A D I D E G M D R A M A G P A K K	
442	GATCGTATTCAATCAATGCGGAACGTGAGATCGTGGCTTACCAGGAAGCAGGTACGCTATTGTTGGACTCGTCTTGAATAATGGATCTACTGTTGCTA	1700
	D R I Q S M R E R E I V A Y H E A G H A I V G L V L E N G S T V R K	
476	AAGTTACCGTTGTTCCAGTGGACGCATCGGTGGTTACATGCTTCTTCCAGATGAAGAAATCATGCAACCAACTAATTTCCATCTTCAAGACCAACT	1800
	V T V V P R G R I G G Y M L A L P D E E I M Q P T N F H L Q D Q L	
	EcoRV	
509	TGCCAGCCTTATGGGTGGACGACTTGGTGAAGAAATGTCTTTGGTGTAGCTACTCCAGGGGCATCAAAATGATATCGAAAAAGCAACACATTGCTCGT	1900
	A S L M G G R L G E E I V F G V A T P G A S N D I E K A T H I A R	
542	TCAATGGTAACTGAATATGGGATGTCTAAGAACTTGGTATGGTATCTTATGAAGGAGACCATCAAGTATTTATGGTCCGACTATGGTCAAACCTAAGA	2000
	S M V T E Y G M S K K L G M V S Y E G D H Q V F I G R D Y G Q T K T	
576	CTTACTCAGAAGCAACTGCTGTTATGATTGATGATGAAGTGCCTGATTCTCGGTGAAGCTTATGACCGTGCTAAGAAAGCAATGAAACACACCGTGA	2100
	Y S E A T A V M I D D E V R R I L G E A Y D R A K E A I E T H R E	
609	GCAACATAAAGCAATTGCGGAAGCTCTGCTTAAATATGAAACACTTGATGCGAAACAAATCATGCTCACTCTTCAAAACAGGAAAAATGCCTGATGAAGCA	2200
	Q H K A I A E A L L K Y E T L D A K Q I M S L F K T G K M P D E A	
642	GCGGCAGCAGAAGTACCAGAACCAAAACATTTGAAGAATCTCTCAAGATGCAAAATGCGAATGTTGATGATTTTTCAACATTAATATCTATAATGGTG	2300
	A A A E V P E P K T F E E S L K D A N A N V D D F S N I N I Y N G D	
676	ATGAAAAACAGATTCTAAACCAGAGAAATAAGGAAAAATCAGAAGATGAACAGCCGAATAAGGCTGTTTTCTTTTTTATGTTTTAGAATAAGT	2400
	E K T D S K P E E N K E K S E D E T A E *	

Fig. 1. For legend see facing page.

sequence is 71% identical to that of *E. coli* FtsH and 31 amino acid residues are identical among all sequences shown in Fig. 2.

Localization of *L. lactis* FtsH in the membrane

A system using Tn5phoA1 to generate protein fusions between the product of a target gene and alkaline phosphatase (AP) has been developed to detect genes encoding membrane and periplasmic proteins in *E. coli* (Manoil & Beckwith, 1985, 1986). Fusion of AP to a periplasmic protein or to a periplasmic domain of a membrane protein is essential for AP activity. Selection for Tn5phoA1 insertions in pLN2 (Table 1, contains the first 441 codons of *L. lactis* ftsH) resulted in six different plasmids (pLN22–pLN27, Table 1) expressing AP activity in *E. coli*. The fusion points between Tn5phoA1 and pLN2 in pLN22–27 were determined by DNA sequencing. All the insertions were located in the ftsH coding region between nucleotide 467 and 663 (codons 64–129) (Fig. 1).

To further analyse the localization of the *L. lactis* FtsH protein in the cell, we carried out Western blotting analysis of fractionated *L. lactis* cell extracts with anti-*E. coli* FtsH serum, which also reacts with FtsH of *L. lactis*. Sonicated cell extracts from *L. lactis* strain MG1363 and DN4302 ftsH::pLN43 (described below) were fractionated into cytoplasmic and membrane fractions by ultracentrifugation. The membrane fraction was washed with 1 M NaCl and separated into supernatant and pellet. In Fig. 3(a) and (b) (lanes 1–3) a SDS-polyacrylamide gel of the various fractions of cell extract of MG1363 and DN4302 blotted to nitrocellulose and gold-stained is shown. No significant differences in the protein patterns of the cytoplasmic or membrane fractions of MG1363 and DN4302 were found (Fig. 3a and b, lanes 1 and 3), whereas, the supernatants of the salt-washed membranes differed in protein patterns (Fig. 3a and b, lanes 2). The anti-*E. coli* FtsH serum only detected FtsH from MG1363 and a truncated form of FtsH from DN4302 in the pellets of the salt-washed membrane fractions (Fig. 3a and b, lanes 6).

It is concluded from the results described above, that FtsH of *L. lactis* is an integral membrane protein spanning the membrane twice with the region flanked by these hydrophobic stretches protruding outside the cell, and that it has a large cytoplasmic carboxy-terminal part with a putative ATP-binding domain (Fig. 4). Such overall features of the *L. lactis* FtsH topology are consistent with those of the *E. coli* homologue, FtsH (Tomoyasu *et al.*,

1993b), except that *L. lactis* FtsH has a larger outside domain than *E. coli* FtsH.

Complementation of Δ ftsH3::kan in *E. coli* with ftsH of *L. lactis*

Since the deduced amino acid sequences of ftsH from *L. lactis* and *E. coli* showed a high degree of identity, we were interested to know whether or not the *L. lactis* ftsH gene could complement a ftsH mutation in *E. coli*.

The *E. coli* strain AR423 Δ ftsH3::kan (pAR171 ftsH rep^{ts} Cm^R) shows temperature-sensitive growth, because the replication of the plasmid pAR171, containing the essential ftsH gene and a chloramphenicol (Cm) resistance marker, is defective at 42 °C (Akiyama *et al.*, 1994a).

The plasmid pLN32 contains the entire *L. lactis* ftsH gene, whereas the plasmids pLN2 and pLN39 contain various parts of the *L. lactis* ftsH gene, all cloned in the vector pBluescript KS+ or pBluescript SK– containing ampicillin resistance (Ap^R) markers (Fig. 5a, Table 1). These plasmids were used to transform strain AR423 by selection for Ap^R at 30 °C. The strains obtained were incubated in LB at 42 °C for 6 h, and then plated on LB agar at 30 °C. The plasmid content of the colonies was tested by streaking on LB agar containing Ap or Cm; and the possession of the Δ ftsH3::kan mutation was tested by streaking on LB agar containing Kan. From transformants containing the entire *L. lactis* ftsH gene on pLN32, Cm^S colonies could be isolated with a frequency of approximately 40–50%, indicating that these had lost pAR171, containing the *E. coli* ftsH wild-type. Such colonies were all Ap^R and Kan^R, indicating that these had retained pLN32, containing the *L. lactis* ftsH gene, and the mutation Δ ftsH3::kan. Colonies of transformants with pLN2 and pLN39, containing only part of *L. lactis* ftsH, were all Ap^R, Cm^R and Kan^R, showing that no loss of pAR171 was obtained. Thus pAR171, and hereby the *E. coli* wild-type ftsH gene, can be lost only from strains containing the complete *L. lactis* ftsH gene. One strain, AR3120 Δ ftsH3::kan (pLN32 *L. lactis* ftsH), was saved for further studies.

Western blots with anti-*E. coli* FtsH serum were performed using extracts of AR423, AR423 containing the plasmids in Fig. 5(a) and AR3120 to detect the gene products of *E. coli* ftsH and/or *L. lactis* ftsH (Fig. 5b). The *E. coli* FtsH protein could be detected in the AR423 derivatives but not in AR3120, whereas the *L. lactis* FtsH protein was detected in AR423(pLN32) and AR3120. No *L. lactis* ftsH product was detected in AR423, AR423(pLN39), AR423(pLN2) or AR423(pKS+). This

Fig. 1. Nucleotide sequence of the *L. lactis* ftsH gene. The numbers to the right indicate nucleotide positions ('hpt, nucleotides 1–72; ftsH, nucleotides 278–2365, 696 codons). The deduced amino acid sequences of 'hpt and ftsH (FtsH) are shown in one-letter code below, with the translation stop codons indicated by asterisks. The numbers to the left indicate the deduced amino acid residue position of FtsH. The putative ribosome binding sites (RBS) and promoter regions (–35, –16, –10) are indicated. Two putative stem-loop structures are underlined. The Tn5phoA1 insertions in pLN22–27 are indicated above the sequence. The EcoRV sites used to construct the integration plasmid pLN43 are shown above the sequence.

Yme1p	MNVSKILVSPITVTNVLRI FAPRLPQIGASLLVQKKWALRSKKFYRFYSEKNSGEMPPKKEADSSGKASNKSTISSIDNSQPPPPSNTNDKT	92
LIftsH	MNNNKQPKQGNFVKNI <u>LMWVILAIVVVVGFNFSSNQSSVDKISYSQLMTKLDGNKI</u> ENVTMQPSDSLITVTGEYKEPVKVGKTNFPLLGNSSEVK	100
EcftsHMAKNLILWLVI <u>AVVLSVFQS</u> FGPSESNRKYDYSTFLQEVNNDQVREARINGREINVT.....KKDSNR	65
Yme1p	KQANVAVSHAMLATREGEANKDLTSPDAQAAFYKLLQSNYPQYVVSFETPGIASSPECMELYMEALQIRHSEADAVRQNLITASSAGAVNPSSASS	192
LIftsH	FOAYIPTDSVVKDIQNAAKSNDVKLSVVQASSSGM <u>WVQILSYIIPMLLVGIFWLMHGGMGARGGGGGGNPMSFGKSRAKQDQGT</u> SKVRFADVAGSEE	200
EcftsH	YTTYIPVQDPKLLD...NLLTKNVKVVGEPPPEPS <u>LLASIFISWFPMLLIGVWIFFMROM</u> ...QGGGGKGAMSFGKSKARMLTEDQIKTTFADVAGCDE	159
Yme1p	SSNQSGYHGNFSPMSYPLGSRKEPLHVVVSESTFTVVSRWVKLLVFGILTYSFSEGFKYITENTTLKSSEVADKS...VDVAKTN.VKFDDVCGCDE	288
TBP-1		QYSDIGGLDK 158
CDC1		GYDDIGGCRK 220
CDC2		TWDDVGLDE 493
Pas1p		KWGDIGALAN 704
NSF		EKMIGGLDK 233
LIftsH	E.KQELVEVDFLKNPK.KYHDLGARIPAGVLLGPPGTGKTLAKAVAGEAGVPFYSI.SGSDFVEMFVGVSASRVDRDLFENAKKTAPS.....II	289
EcftsH	A.KEEVAELVEYLREPS.RFQKLGKIPKGVLMVGGPGTGKTLAKAIAAGEAKVPFTI.SGSDFVEMFVGVSASRVDRDMFEQAKKAAPC.....II	248
Yme1p	A.RAELEEIVDFLKDPT.KYESLGGKLPKGVLLTGPPGTGKTLARATAGEAGVDFFM.SGSEFDEVYVGVGAKRIRDLFAQARSRAPE.....II	377
TBP-1	Q.IQELVEAIVLPNNHKEKFENLGIQPPKGVLMYGGPGTGKTLARACAAQTATFLK.AGPQLVQMFIGDGAKLVDAFALAKEKAPS.....II	248
CDC1	Q.MAQIREMVELPLRHPQLFKAIGIKPPRGVLMYGGPGTGKTLARAVANETGAFFFLI.NGPEVMSKMGASESNLRKAFEEAEKNAPA.....II	310
CDC2	I.KEELKETVEYVPLHPDQYTKFGLSPSKGVLFYGGPGTGKTLAKAVATEVSANFISV.KGPELLSMWYGESESNIRDFDKARAAAPT.....VV	583
Pas1p	A.KDVLLETLEVPKYEPFVNCPLRLRSGLLYGYPGCGKTLASAVAQCGCLNFISV.KGPEILNKFIGASEQNIRELFERAAQSVKPC.....IL	794
NSF	EFSDIFRRAFASRVFPEIVEQMGCKHVKGILLYGPPGCGKTLARQIGKMLNAREPKVVGPEILNKYVGESEANIRKLFADAEQRRLGANSGLHI	333
LIftsH	FIDEIDAVGRQRGAGLGGGNDEREQTLNQLLVEMDGFQDDGNSVIVIAATNRSDVLDPALLRPGRFDRKVLVGAPDVKGREAVLKVHA.....KN.K	380
EcftsH	FIDEIDAVGRQRGAGLGGGNDEREQTLNQLLVEMDGFEGN.EGIIIVIAATNRSDVLDPALLRPGRFDRQVVGLPDVRGREQILKVHM.....RR.V	338
Yme1p	FIDEIDAIGGKRN...KQAYAKQTLNQLLVELDGFSQT.SGIIIGATNPFALDKALTRPGRFDRKVVNDLPDVRGRADILKHM.....KK.I	464
TBP-1	FIDEIDAIGTKRFDSEKAGDREVORTMLELLNQLDGFQPTQ.VKVIAATNRVDILDPALLRSGRLDRKIEFPMPNEEARARIMQIHS.....RK.M	338
CDC1	FIDEIDSIAPKRDKTNG...EVERRVVSQLLTMDGMKARSN.VVIAATNRPNIDPALRRFRGRFDREVDIGIPDATGRLVLRITHT.....KN.M	397
CDC2	FIDEIDSIKARGGSLDAGGASDRVVNQLLTMDGMNAKKN.VFVIGATNRPDIDPAILRGRDLQILYVLPDENARLSILNAQL.....RK.T	673
Pas1p	FFDEFDISIAPKRGH...DSTGVTDVVNQLLTMDGAEGLDG.VYLAATSRPDIDALLRPGRLDKSVICNIPTESERLDILQAVNSKDDTGAKKF	890
NSF	IFDEIDAICKQRG.SMAGSTGVHDTVVNQLLSKIDGVEQLNN.ILVIGTNRPLDIDEALLRPGRLVKMEIGLPDEKGRQLIHLIHTARM.....RGHQ	426
LIftsH	PLASVDLHNVAQTQPGYVGADLENVLNEAALVAARONKKEINAADIDEGMDRAMAGPAKKDRIQSMREREIVAYHEAGHAIVGLVLENGSTVRKVTVP	480
EcftsH	PLAPDIDAIIARGTPGFGADLANLVNEAALFAARGNRVVSMEFEKAKDKIMMGAERRSMVMTEAQKESTAYHEAGHAIGRLVPENDPVHKVTIIP	438
Yme1p	TLADNVDPITIIARGTPGLSGAELANLVNQAAVYACQKNVSVDMSHFEWAKDKILMGAERKTMVLDAARKATAFHEAGHAIMAKYNGATPLYKATILP	564
TBP-1	NVSPDVNVEELARCTDDFNGAQCKAVCVEA	368
CDC1	KLADDVDLEALAEATHGYVGADIASLCSEA	427
CDC2	PLEPGLLETAIAKATQGFSGADLLYIVQRA	703
Pas1p	ALEKNADLKLIAEKTAGFSGADLGLCYNA	920
NSF	LLSADVDIKELAVETKNFSGAELEGLVRAA	456
LIftsH	RGRIGGYMLALPDEEIMOPTNFHLQDQLASLMGGRLGEEIVFGVA..TPGASNDIEKATHIARSMVTEYGMSSKLGMSVY.EGDHQVFIGRDYGTQKTS	577
EcftsH	RGRALGVTFFLPEGDAISASRQKLESQISTLYGGRLAEEIYGPEHVSSTGASNDIKVATNLARNMVTQWGFSEKLGPLLYAEEEGEVFLGRSVAKAKHMS	538
Yme1p	RGRALGITFQLPEMDKVDITKRECQARLDVCMGGKIAEELIYGKNDTSGCGSDLSQATGTARAMVTQYGMSSDDVGPVNLSEN....WESWS.NKIRD...	657
LIftsH	EATAVMIDDEVRRILGEAYDRAKEAIEHREQHKAIAEALLKYETLDAQKIMSLFKTGKMPDEAAAAEVPKPTFEESLKDANANVDDF.SNINIYNGDE	676
EcftsH	DETARIIDQEVKALIERNYNRARQLLTDNMDILHAMKDALMYETIDAPQIDDL.....ARRDVRPPAGWEEP..GASNNSGDNGSPKAPRPVDE	627
Yme1pIADNEVIELLKDSERARRLLTKKNVELHRLAAGLIEYETLDAHEIEQV.....CKGEKLD....KLKTSNTVVEGPDSDERKDIKF	736
LIftsH	KTDSKPEENKEKSEDETAE	695
EcftsH	PRTNPG.NTM.SEO	640
Yme1p	DKPKIP...TMLNA	747

Fig. 2. Amino acid sequence alignment of *L. lactis* (Li) FtsH, *E. coli* (Ec) FtsH, Yme1p and the conserved domains found in *L. lactis* FtsH (residues 191–410); TBP-1 (Nelbock *et al.*, 1990); CDC48p (CDC1, CDC2; CDC48p contains two conserved domains) (Fröhlich *et al.*, 1991); Pas1p (Erdmann *et al.*, 1991); and NSF (Wilson *et al.*, 1989). S4 of the 26S protease, MSS1 and SUG1 resembling TBP-1 (Dubiel *et al.*, 1992; Shibuya *et al.*, 1992; Swaffield *et al.*, 1992), VCP and p97 resembling CDC48p (Koller & Brownstein, 1987; Peters *et al.*, 1990), and Sec18p resembling NSF (Eakle *et al.*, 1988) also contain the conserved domain (not shown). Identical amino acid residues found in these sequences are indicated in boldface type above the sequences. Identical amino acid residues of *L. lactis* FtsH, *E. coli* FtsH and Yme1p are indicated by asterisks. The two putative membrane-spanning sequences of *L. lactis* FtsH and *E. coli* FtsH are underlined.

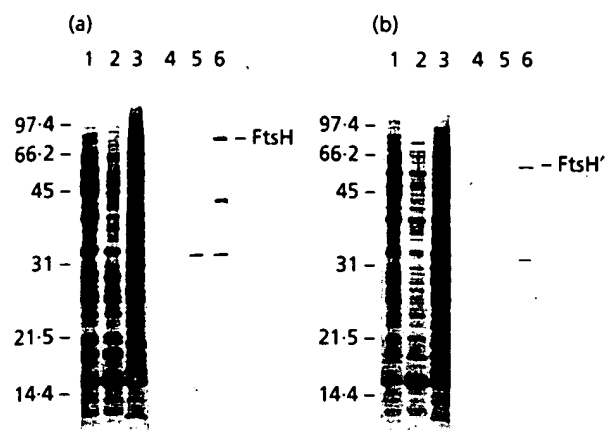


Fig. 3. Localization of *L. lactis* FtsH in cell fractions. Fractionation of extracts from MG1363 and DN4302, and treatment of membrane fractions with 1 M NaCl were done as described in Methods. Electrophoresis of the cell fractions on an SDS-polyacrylamide gel (a and b) was done, blotted to nitrocellulose and gold-stained (lanes 1–3) or subjected to Western blotting with anti-*E. coli* FtsH serum (lanes 4–6). Positions of molecular mass reference markers, FtsH and a truncated form of FtsH (FtsH') are indicated. All other bands are a result of unspecific binding of the secondary anti-serum used (control not shown). (a) Lanes: 1 and 4, cytoplasmic fraction of MG1363; 2 and 5, supernatant derived from membrane fraction of MG1363 washed with 1 M NaCl; 3 and 6, pellet derived from membrane fraction of MG1363 washed with 1 M NaCl. (b) Lanes: same as (a) except extract from DN4302 was used.

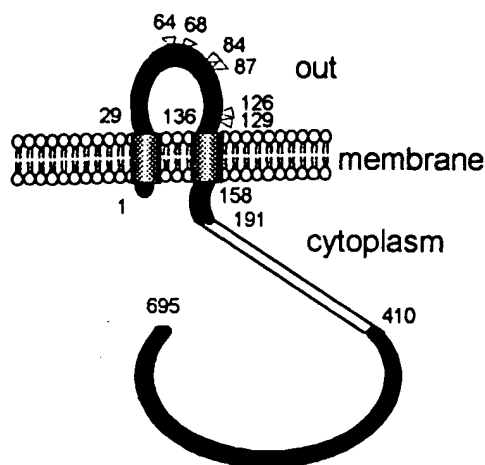


Fig. 4. Deduced topology of the *L. lactis* FtsH protein. Numbers indicate amino acid residue positions. Shaded boxes illustrate putative membrane-spanning regions. The encoded FtsH-PhoA fusions of pLN22–27 that results in AP activity are fused at positions indicated by triangles. The region with similarity to the conserved region of the AAA-protein family is indicated by an open box. This region contains the ATP binding motifs.

demonstrates that AR3120 was able to grow without the essential *E. coli* ftsH gene product when containing the *L. lactis* ftsH gene product. The plasmid pLN2 encodes a truncated *L. lactis* FtsH protein containing the epitope for the anti-*E. coli* FtsH serum, when transcribed and

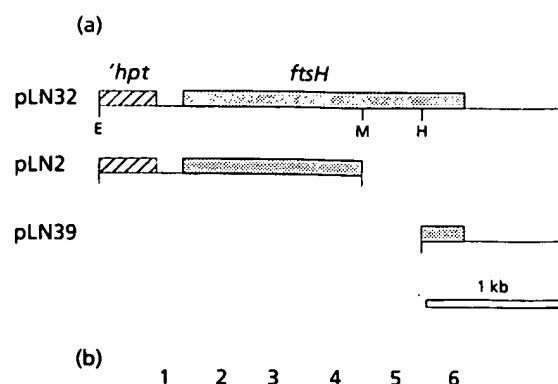


Fig. 5. Immunological detection of *L. lactis* FtsH and *E. coli* FtsH. (a) The lines represent *L. lactis* chromosomal DNA fragments cloned in pBluescript KS+ or SK-. The restriction enzyme sites shown are: E, *EcoRI*; H, *HindIII*; M, *MboI*. The location of the *L. lactis* 'hpt' and ftsH genes is indicated by hatched and shaded areas, respectively. (b) Western blot. The FtsH proteins of *L. lactis* (LI) and *E. coli* (Ec) were detected using anti-*E. coli* FtsH serum. The positions of *L. lactis* FtsH and *E. coli* FtsH are indicated to the left. Lanes: 1, AR423(pKS+); 2, AR423(pLN39); 3, AR423(pLN2); 4, AR3120; 5, AR423(pLN32); 6, AR423. The molecular mass reference markers are indicated on the right.

translated *in vitro* (Nilsson & Lauridsen, 1992; data not shown). This truncated FtsH was not detected by the anti-*E. coli* FtsH serum in the Western blot, possibly due to rapid degradation *in vivo*.

E. coli AR3120 showed temperature-sensitive growth in that it could grow at 30 °C and 37 °C but not at 42 °C. A *B. subtilis* mutant with a salt-sensitive phenotype has been shown to be impaired in a putative homologue of ftsH (Geisler & Schumann, 1993). AR423, AR423(pLN32) and AR3120 were tested for growth on LB agar plates containing 4% (w/v) NaCl. AR423 and AR423(pLN32), but not AR3120, were able to grow on this medium.

Construction of a *L. lactis* ftsH mutant

A 1.3 kb *EcoRV* fragment of pLN32, containing an internal part of *L. lactis* ftsH (Fig. 1), was cloned into the *L. lactis* integration vector pV2 giving pLN43 (Table 1).

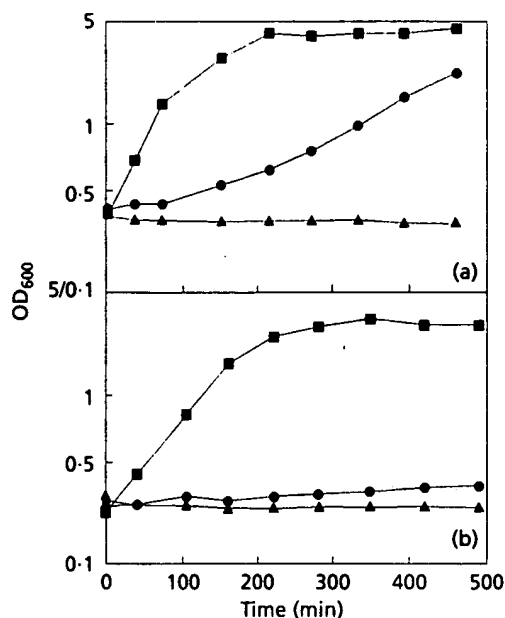


Fig. 6. Growth of DN4302 and MG1363 at various NaCl concentrations. MG1363 (a) and DN4302 (b) were grown exponentially in M17 medium and then diluted twofold in M17 medium containing 0% (■), 4% (●) or 7% (▲) (w/v) NaCl (final concentrations).

Transformation of pLN43 into *L. lactis* strain MG1363, selecting for erythromycin resistance, resulted in strain DN4302 *ftsH::pLN43*. The integration of pLN43 into *ftsH* of DN4302 was verified by Southern hybridization analysis (not shown). The construction of DN4302 indicates that the *ftsH* gene is not essential in *L. lactis* or that the truncated form of FtsH encoded by the disrupted *ftsH* gene (Fig. 3b, lane 6) is enough to retain any essential function. DN4302 and MG1363 were tested for growth on M17-agar plates containing 4% or 1% NaCl. MG1363 could grow at both 4% and 1% NaCl, whereas DN4302 grew slowly at 1% and not at all at 4% NaCl. Fig. 6 shows the growth of DN4302 and MG1363 at various salt concentrations in M17 broth. MG1363 grew at 4% NaCl after a lag phase of about 2 h, whereas DN4302 at 4% NaCl showed almost no growth. No growth of either strains occurred with 7% NaCl. DN4302 and MG1363 were also tested for growth on M17-agar plates containing 0.5 M sucrose. Both strains appeared to grow equally well on this medium.

Growth at 38 °C, 30 °C and 16 °C on M17-agar plates was also tested. DN4302 only grew at 30 °C, whereas MG1363 grew at all temperatures. However, incubating DN4302 anaerobically restored growth at 38 °C.

Homologous genes in other bacteria

A 1.0 kb *Hind*III–*Kpn*I fragment (nucleotides 667–1600, Fig. 1) was used as probe in Southern hybridization analysis with *Sca*I-digested chromosomal DNA from

various bacterial strains (Fig. 7). Single chromosomal bands can be detected from the Gram-positive bacteria *L. lactis*, *B. subtilis*, *Leuconostoc* sp. and *Lactobacillus* sp. (Fig. 7, lanes 1, 3–8). With *E. coli* DNA only a very faint band was detected (Fig. 7, lane 2). Under the same experimental conditions, homology to *hpt*, using a *hpt* probe, was only detected in *L. lactis* (results not shown).

DISCUSSION

The *ftsH* gene of *L. lactis* was cloned and shown to encode a transmembrane protein with putative ATPase activity. The deduced amino acid sequence indicates that FtsH belongs to the AAA-protein family of putative ATPases containing several members of eukaryotic proteins (Erdmann *et al.*, 1991; Kunau *et al.*, 1993) and also includes the membrane protein FtsH of *E. coli* (Tomoyasu *et al.*, 1993a, b). FtsH from *L. lactis* and *E. coli* seem to be homologous proteins based on the high similarity of the amino acid sequences and that the *L. lactis* *ftsH* gene can complement certain defects of an *E. coli* Δ *ftsH3::kan* strain. Southern hybridization analysis of various Gram-positive bacteria using *L. lactis* *ftsH* as probe suggests that homologous genes exist. In *B. subtilis* a putative *ftsH* gene has been located on the chromosome of *B. subtilis* adjacent to the *hpt* gene (Geisler & Schumann, 1993; Ogasawara *et al.*, 1994), indicating a similar arrangement of *hpt* and *ftsH* as in *L. lactis*. It seems from these results that *ftsH* genes are widely conserved in bacteria.

The function of FtsH is unknown. Our *L. lactis* *ftsH* mutant and the *B. subtilis* *ftsH* mutant (Geisler & Schumann, 1993) were constructed by a Campbell-type integration with an internal gene fragment. This should inactivate the genes. In *E. coli*, *ftsH* is essential for growth. Since the resulting mutants are viable, it is possible that the *ftsH* genes are not essential in *L. lactis* and *B. subtilis*. However, in at least *L. lactis*, the truncated form of FtsH produced by the mutant DN4302 may still retain essential functions.

The *L. lactis* *ftsH* mutant DN4302 is impaired in salt tolerance and other stress responses, and shows a different pattern of membrane-associated proteins compared to the wild-type strain (Fig. 3, lanes 2). These phenotypes could be explained by an improper assembly of membrane proteins, some necessary for the salt tolerance, caused by the *ftsH* mutations in *L. lactis*. In *E. coli* the maturation of penicillin binding protein 3 (PBP3) and β -lactamase is dependent on FtsH function, in that post-translational processing at the C-terminal part of PBP3 seems to be defective and accumulation of the plasmid-encoded precursor of β -lactamase in the cytoplasm was observed in the thermosensitive *ftsH1* mutant of *E. coli* (Begg *et al.*, 1992; Tomoyasu *et al.*, 1993a). Analysis of several newly constructed *E. coli* *ftsH* mutants encoding FtsH variants, including C-terminally truncated forms with dominant phenotypes, suggests that FtsH is involved in assembly/folding of proteins into and through the membrane and that FtsH is needed to assure efficient stop-transfer of some membrane proteins (Akiyama *et al.*, 1994a, b).

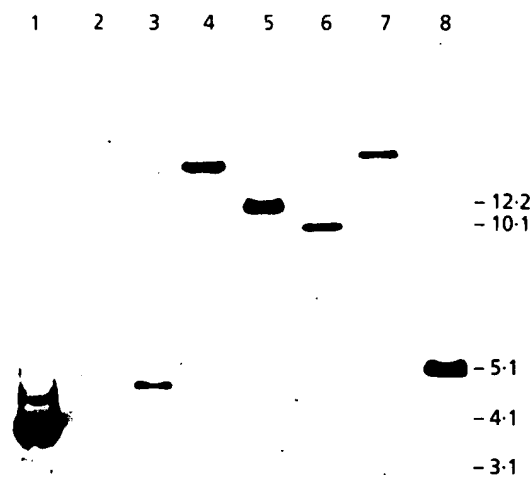


Fig. 7. Southern hybridization of an internal part of the *L. lactis* *ftsH* gene to chromosomal DNA from various bacteria. Lanes: 1, *L. lactis* CHCC285; 2, *E. coli* XL-1 Blue; 3, *B. subtilis* 168; 4, *Leuconostoc mesenteroides* DB1165; 5, *Leu. denos* LOP89005; 6, *Lactobacillus plantarum* LP2T1G; 7, *Lactob. acidophilus* LA2753; 8, *Lactob. lactis* CH-2. Molecular mass markers are shown on the right in kb.

However, how FtsH is functionally related to these phenotypes is not known. Recently it was shown that a *hflB* mutation in *E. coli* causing increased lysogenization frequencies of bacteriophage λ through a reduced degradation of the ϵ II protein, is located in *ftsH*, and the HflB/FtsH protein has been suggested to be an energy-dependent chaperone and/or protease (Herman *et al.*, 1993). Other members of the AAA-protein family are also involved in proteolysis, e.g. subunit 4 of the human ATP-dependent 26S protease, and it was proposed that other eukaryotic members such as Tbp1, Sug1 and MSS1 are also subunits of the 26S protease (Dubiel *et al.*, 1992). Taken together FtsH might function as a chaperone and/or a protease although no direct biochemical evidence for these functions has been reported so far. Comparative biochemical and genetic analysis of FtsH from *L. lactis* and *E. coli* will provide better understanding of the biological significance of FtsH, if it is a chaperone or a protease, and what are its substrates.

ACKNOWLEDGEMENTS

We are pleased to thank: Eric Johansen, Egon Bech Hansen (Chr. Hansen's Laboratorium, Hørsholm, Denmark) and Per Nygaard (Enzyme division, Copenhagen University, Denmark) for careful reading of the manuscript; Henrik Ipsen (ALK, Denmark) for discussion; Eric Johansen for providing chromosomal DNA from *Leuconostoc* spp. and *Lactobacillus* spp.; Svend E. W. Hansen for the integration vector pV2; and Hans Henrik Saxild (Danish Technical University, Lyngby,

Denmark) for *Bacillus subtilis* 168. We are also grateful to Michael Blom Sørensen (Carlsberg Laboratories, Denmark) for the provision of customized synthetic oligonucleotides and Thomas Laybourn and Lotte Friberg (ALK, Denmark) for technical assistance with the Western blots. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and the Lundbeck Foundation in Denmark.

REFERENCES

- Akiyama, Y., Ogura, T. & Ito, K. (1994a). Involvement of FtsH in protein assembly into and through the membrane. I. Mutations that reduce retention efficiency of a cytoplasmic reporter. *J Biol Chem* **269**, 5218–5224.
- Akiyama, Y., Shirai, Y. & Ito, K. (1994b). Involvement of FtsH in protein assembly into and through the membrane. II. Dominant mutations affecting FtsH function. *J Biol Chem* **269**, 5225–5229.
- Begg, K. J., Tomoyasu, T., Donachie, W. D., Khattar, M., Niki, H., Yamanaka, K., Hiraga, S. & Ogura, T. (1992). *Escherichia coli* mutant Y16 is a double mutant carrying thermosensitive *ftsH* and *ftsI* mutations. *J Bacteriol* **174**, 2416–2417.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Clark, D. J. & Maaløe, O. (1967). DNA replication and the division cycle in *Escherichia coli*. *J Mol Biol* **23**, 99–112.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Dubiel, W., Ferrell, K., Pratt, G. & Rechsteiner, M. (1992). Subunit 4 of the 26S protease is a member of a novel eucaryotic ATPase family. *J Biol Chem* **267**, 22699–22702.
- Eakle, K. A., Bernstein, M. & Emr, S. D. (1988). Characterization of a component of the yeast secretion machinery: identification of the *SEC18* gene product. *Mol Cell Biol* **8**, 4098–4109.
- Erdmann, R., Wiebel, F. F., Flessau, A., Rytka, J., Beyer, A., Fröhlich, K. & Kunau, W. (1991). *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell* **64**, 499–510.
- Fröhlich, K., Fries, H., Rüdiger, M., Erdmann, R., Botstein, D. & Mecke, D. (1991). Yeast cell cycle protein CDC48p shows full-length homology to the mammalian protein VCP and is a member of a protein family involved in secretion, peroxisome formation, and gene expression. *J Cell Biol* **114**, 443–453.
- Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**, 1–9.
- Geisler, U. & Schumann, W. (1993). Isolation of stress mutants of *Bacillus subtilis* by a novel genetic method. *FEMS Microbiol Lett* **108**, 251–254.
- Gutierrez, C., Barondess, J., Manoil, C. & Beckwith, J. (1987). The use of transposon Tn ϕ 4 to detect genes for cell envelope proteins subject to a common regulatory stimulus. *J Mol Biol* **195**, 289–297.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Henkin, T. M., Donnelly, C. E. & Sonenshein, A. L. (1988). Mutations in the spacer region of a *Bacillus subtilis* promoter. In *Genetics and Biotechnology of Bacilli*, Vol. 2, pp. 63–67. Edited by A. T. Ganesan & J. A. Hoch. San Diego: Academic Press.
- Herman, C., Ogura, T., Tomoyasu, T., Hiraga, S., Akiyama, Y., Ito, K., Thomas, R., D'Ari, R. & Boulloc, P. (1993). Cell growth and λ

- development controlled by the same essential *Escherichia coli* gene, *ftsH/hfb*. *Proc Natl Acad Sci USA* 90, 10861–10865.
- Holo, H. & Nes, I. F. (1989). High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* 55, 3119–3123.
- Ipsen, H. & Larsen, J. N. (1988). Detection of antigen-specific IgE antibodies in sera from allergic patients by SDS-PAGE immunoblotting and crossed radio-immunoelectrophoresis. In *Handbook of Immunoblotting of Proteins*, Vol. II, pp. 159–166. Edited by O. Bjerrum & N. H. Heegaard. Boca Raton: CRC Press.
- Jochimsen, B., Nygaard, P. & Vestergaard, T. (1975). Location on the chromosome of *Escherichia coli* of genes governing purine metabolism. *Mol & Gen Genet* 143, 85–91.
- Johansen, E. & Kibenich, A. (1992). Characterization of *Leuconostoc* isolates from commercial mixed-strain mesophilic starter cultures. *J Dairy Sci* 75, 1186–1191.
- Koivula, T., Sibakov, M. & Palva, I. (1991). Isolation and characterization of *Lactococcus lactis* subsp. *lactis* promoters. *Appl Environ Microbiol* 57, 333–340.
- Koller, K. J. & Brownstein, M. J. (1987). Use of a cDNA clone to identify a supposed precursor protein containing valosin. *Nature* 325, 542–545.
- Kunau, W. H., Beyer, A., Franken, T., Götte, K., Marzioch, M., Saidowsky, J., Skaletz-Rorowski, A. & Wiebel, F. F. (1993). Two complementary approaches to study peroxisome biogenesis in *Saccharomyces cerevisiae*: forward and reversed genetics. *Biochimie* 75, 209–224.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157, 105–132.
- Larsen, J. N., Ströman, P. & Ipsen, H. (1992). PCR based cloning and sequencing of genes encoding the tree pollen major allergen *Car b 1* from *Carpinus betulus* (hornbeam). *Mol Immunol* 29, 703–711.
- Ludwig, W., Seewaldt, E., Klipper-Balz, R., Schleifer, K. H., Magrum, L., Woese, C. R., Fox, G. E. & Stackebrandt, E. (1985). The phylogenetic position of *Streptococci* and *Enterococcus*. *J Gen Microbiol* 131, 543–551.
- Mandel, M. & Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J Mol Biol* 53, 159–162.
- Manoil, C. & Beckwith, J. (1985). *TnpA*: a transposon for protein export signals. *Proc Natl Acad Sci USA* 82, 8129–8133.
- Manoil, C. & Beckwith, J. (1986). A genetic approach to analyzing membrane protein topology. *Science* 233, 1403–1408.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moran, C. P., Jr, Lang, N., Le Grice, F. S. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. & Losick, R. (1982). Nucleotide sequence that signals the initiation of transcription and translation in *Bacillus subtilis*. *Mol & Gen Genet* 186, 339–346.
- Nelbock, P., Dillon, P. J., Perkins, A. & Rosen, C. A. (1990). A cDNA for a protein that interacts with the human immunodeficiency virus Tat transactivator. *Science* 248, 1650–1653.
- Nilsson, D. & Johansen, E. (1994). A conserved sequence in tRNA and rRNA promoters of *Lactococcus lactis*. *Biochim Biophys Acta* (in press).
- Nilsson, D. & Lauridsen, A. A. (1992). Isolation of purine auxotrophic mutants of *Lactococcus lactis* and characterization of the gene *bpt* encoding hypoxanthine guanine phosphoribosyl-transferase. *Mol & Gen Genet* 235, 359–364.
- Ogasawara, N., Nakai, S. & Yoshikawa, H. (1994). Systematic sequencing of the 180 kilobase region of the *Bacillus subtilis* chromosome containing the replication origin. *DNA Res* 1, 1–14.
- Ogura, T., Tomoyasu, T., Yuki, T., Morimura, S., Begg, K. J., Donachie, W. D., Mori, H., Niki, H. & Hiraga, S. (1991). Structure and function of the *ftsH* gene in *Escherichia coli*. *Res Microbiol* 142, 279–282.
- Peters, J.-M., Walsh, M. J. & Franke, W. W. (1990). An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative fusion proteins Sec18p and NSF. *EMBO J* 9, 1757–1767.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74, 5463–5467.
- Shibuya, H., Irie, K., Ninomiya-Tsuji, J., Goebel, M., Taniguchi, T. & Matsumoto, K. (1992). New human gene encoding a positive modulator of HIV Tat-mediated transactivation. *Nature* 357, 700–702.
- Shine, J. & Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc Natl Acad Sci USA* 71, 1342–1346.
- Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Swaffield, J. C., Bromberg, J. F. & Johnston, S. A. (1992). Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. *Nature* 357, 698–700.
- Thorsness, P. E., White, K. H. & Fox, T. D. (1993). Inactivation of *YME1*, a member of the *ftsH-SEC18-PAS1-cdc48* family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae*. *Mol Cell Biol* 13, 5418–5426.
- Tomoyasu, T., Yuki, T., Morimura, S., Mori, H., Yamanaka, K., Niki, H., Hiraga, S. & Ogura, T. (1993a). The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* 175, 1344–1351.
- Tomoyasu, T., Yamanaka, K., Murata, K., Suzuki, T., Boulloc, P., Kato, A., Niki, H., Hiraga, S. & Ogura, T. (1993b). Topology and subcellular localization of FtsH protein in *Escherichia coli*. *J Bacteriol* 175, 1352–1357.
- Walker, J. E., Saratse, M., Runswick, M. J. & Gay, N. J. (1982). Distantly related sequences in the α - and β -subunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1, 945–951.
- Wilson, D. W., Wilcox, C. A., Flynn, G. C., Chen, E., Kuang, W., Henzel, W. J., Block, M. R., Ullrich, A. & Rothman, J. E. (1989). A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339, 355–359.
- von Wright, A., Tynkkynen, S. & Suominen, M. (1987). Cloning of a *Streptococcus lactis* subsp. *lactis* chromosomal fragment associated with the ability to grow in milk. *Appl Environ Microbiol* 53, 1584–1688.

Received 14 March 1994; revised 31 May 1994; accepted 8 June 1994.

From: Steadman, David (AU1652)
Sent: Wednesday, February 13, 2002 1:57 PM
To: STIC-ILL
Subject: Literature/ILL request

Art Unit: 1652
Office: 10D-04
Mailbox: 10C-01 M3
Case Serial #:09/720,096

Please provide the following reference:

1) J Biol Chem 1990 Nov 5;265(31):18776-9
Saturation site-directed mutagenesis of thymidylate synthase.
~~Clinic S, Ruiz-Perez L, Gonzalez-Pacanowska D, Prapunwattana P, Cho SW, Stroud R, Santi DV.~~

2) Microbiology 1994 Oct;140 (Pt 10):2601-10
A Lactococcus lactis gene encodes a membrane protein with putative ATPase activity that is homologous to the essential Escherichia coli ftsH gene product.
Nilsson D, Lauridsen AA, Tomoyasu T, Ogura T.

Thank you very much,
David J. Steadman
Art Unit 1652
CM1, 10D-04
308-3934

Saturation Site-directed Mutagenesis of Thymidylate Synthase*

(Received for publication, May 18, 1990)

Shane Climiet†, Luis Ruiz-Perez‡§, Dolores Gonzalez-Pacanowska§, Phisit Prapunwattana†, Sung-Woo Cho†, Robert Stroud†, and Daniel V. Santi‡¶

From the †Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0448 and the §Institute Lopez-Neyra of Parasitology, Consejo Superior de Investigaciones Cientificas, Granada, 18001 Spain

We have subjected 12 different codons of a synthetic *Lactobacillus casei* thymidylate synthase (TS) gene to saturation site-directed mutagenesis to create amino acid "replacement sets" at each of those positions. The target residues were chosen because they are highly conserved and because they are important for the structure and function of the protein as indicated by solution and structural studies. The mutagenesis procedure involved excision of a fragment of the synthetic gene containing the target codon, followed by its replacement with a mixture of oligonucleotides which code for all 20 amino acids and the amber stop codon. TS mutants were identified by DNA sequencing, and catalytically active mutants were identified by genetic complementation using a *Thy*⁻ strain of *Escherichia coli*. Only 3 of the 12 target amino acids examined were essential for TS activity; and of the 125 total mutants identified, 57 were catalytically active. These results point to a high degree of plasticity of TS in accommodating function with structural change.

Thymidylate synthase (TS)¹ catalyzes the conversion of 2'-deoxyuridylyl (dUMP) and 5,10-methylenetetrahydrofolate to 2'-deoxythymidylate (dTMP) and dihydrofolate. TS is the sole *de novo* source of dTMP, and there has been much interest in studies of its structure, function, and inhibition. In part, the interest in TS results from the large amount of information on this enzyme which now exists. The enzymology of TS has been extensively studied, and details about its mechanism and inhibition are well understood (1). Also, the primary sequences of TS from some 16 sources have been determined and have revealed that it is the one of most conserved proteins known (2); the similarity among TS sequences implies a functional importance for the conserved residues. Finally, the x-ray structures of the *Lactobacillus casei* TS-P_i complex and the *Escherichia coli* TS-dUMP-10-propargyl-5,8-dideazafolate (CB3717) ternary complex have provided insight about the possible roles of specific residues of the protein (3-5).

Thus far, mutations of key residues of TS have provided unexpected results. His¹⁰⁹, a putative general base catalyst (6,

7), and Arg¹⁷⁹, a substrate-binding residue (8), could each be substituted by several different residues without large effects on binding or catalysis; even the Cys involved in nucleophilic catalysis could be modified to Ser and still retain activity, albeit severely diminished (9). From such observations, we became cautious about directing our efforts toward the study of one or a few mutants. Instead, we adopted a plan to produce all possible mutants at a residue of interest and developed screening methods for various properties to reveal mutants of interest.

In this report, we describe the strategy and preliminary results of an approach to understanding the structure of TS by saturation site-directed mutagenesis. The mutations were constructed by cassette mutagenesis using an *E. coli* expression vector that carries a synthetic *L. casei* TS gene with over 30 unique restriction sites (7). The mutagenic DNA cassettes contained mixtures of 32 codons that encode 20 amino acids and the amber (TAG) stop codon at the target site (10-12). "Replacements sets" were constructed in which each of 12 target amino acids were replaced by a large number of substitutions, and a complementation assay was used to rapidly screen for mutants that were catalytically active and thus of more interest for further study.²

MATERIALS AND METHODS

Restriction endonucleases and T4 ligase were purchased from New England BioLabs and Bethesda Research Laboratories and were used as recommended. The Sequenase DNA sequencing kit was purchased from U. S. Biochemical Corp. The *E. coli* strains used were TB-1 (φ80lacZΔM15; ara, Δ(lac-proAB), rpsL, hsdR) from T. O. Baldwin (Texas A & M, College Station, TX) and *Thy*⁻ 2913 (ΔthyA572) from R. Thompson (University of Glasgow, United Kingdom). Oligonucleotides were synthesized at the University of California-San Francisco Biomolecular Resource Center using an Applied Biosystems 380B DNA synthesizer; tritylated oligonucleotide mixtures were purified and de-tritylated on Du Pont-New England Nuclear NEN-SORB Prep cartridges using the recommendations of the manufacturer. General methods for plasmid purification, subcloning, and bacterial transformation were as described (13).

In vitro cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9 (7). In some cases, pSCTS9 was first modified by substituting a 19-base noncoding sequence containing unique *NotI* and *SphI* sites for the fragment containing the target codon; this eliminates wild-type sequences and allows for selective destruction of the parent plasmid and resultant increase in mutagenesis efficiency (14). Methods for the construction and characterization of the TS mutants were as described (7). Short fragments of the TS gene containing the target codon were replaced with a synthetic DNA duplex presenting a mixture of all four bases at the first and second positions of the target codon and a mixture of G and C at the third position. The plasmids from the ligation mixture were used to trans-

* This work was supported by United States Public Health Service Grant CA14394 and the TDR/Rockefeller Foundation Joint Venture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Senior Fulbright/Spanish Ministry of Education and Science Scholar.

‡ To whom reprint requests should be addressed.

§ The abbreviation used is: TS, thymidylate synthase.

² The use of a series of amber tRNA suppressors and genetic complementation to construct and characterize a large number of mutants of *E. coli* TS was reported after this manuscript was submitted (16).

form *E. coli* TB-1, and plasmid pools were transformed into *E. coli* Thy⁻ χ 2913. Individual plasmids were twice re-transformed into χ 2913 and sequenced. Catalytically active substitutions were identified by screening for the ability of Thy⁻ χ 2913 cells harboring the mutant plasmids to grow on minimal agar lacking Thd and were again characterized by DNA sequencing.

RESULTS AND DISCUSSION

The amino acids targeted for mutagenesis were chosen because they are highly conserved and because they have putative roles in substrate or cofactor binding or in catalysis, as determined by solution or structural studies (Fig. 1). The strategy used in the construction and characterization of the replacement sets is outlined in Fig. 2. Following ligation of oligonucleotide mixtures and initial transformation, colonies were pooled, and the mutagenized plasmid DNA pool was recovered and used to transform *E. coli* Thy⁻ χ 2913 to ampicillin resistance. Individual mutants were identified by DNA sequencing, and the resulting plasmids were used to re-transform χ 2913. Plasmid DNA from the secondary χ 2913 transformants was again sequenced and tested for TS activity as described below. Passage of the mutagenized DNA mixtures through several rounds of transformation ensured segregation and/or repair of the heteroduplex DNA molecules that were created during the construction of the mutants. It was not practical to identify all possible mutants in a replacement set by this strategy, which would require sequencing of over 160 clones to obtain a 95% confidence level of obtaining a complete set. Generally, 20–30 isolates of a replacement set were sequenced to give 10–15 different mutants of a given residue.

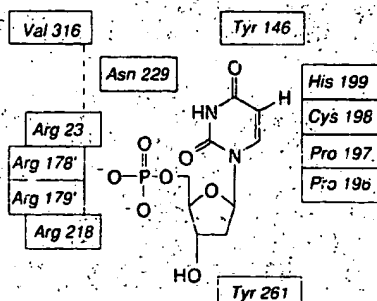


FIG. 1. Amino acid residues of TS targeted for mutagenesis depicted near the sites of interaction with dUMP. --- hydrogen bond between Val³¹⁶ and Arg²³.

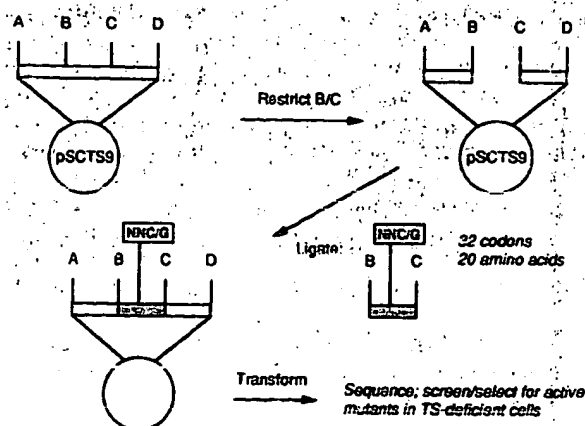


FIG. 2. Strategy for saturation site-directed mutagenesis of synthetic TS gene and identification of catalytically active mutants. A fragment of the TS gene between any two restriction sites is removed and replaced by a mixture of oligonucleotides containing NNG/C at the target codon. The resultant plasmids are sequenced and screened in *E. coli* Thy⁻ cells for catalytic activity.

An important step in our strategy involves the identification of catalytically active mutants by genetic complementation of *E. coli* χ 2913, which is deficient in TS. Individual mutant isolates were grown under nonselective conditions and then patched onto minimal agar lacking Thd. Cell growth in the absence of exogenous Thd identifies mutants that express catalytically active TS. Mutants were scored for full growth, weak growth, or no growth. A comparison of the specific activity (data not shown) of TS in crude extracts from a variety of mutants indicated that a specific activity of $\sim 0.002 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ($0.001\text{--}0.005 \mu\text{mol min}^{-1} \text{mg}^{-1}$) is necessary for complementation of χ 2913; this corresponds to $\sim 1\%$ of the activity of cells harboring the parent plasmid pSCT59.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell extracts showed that TS was expressed at levels of 5–30% of the total soluble protein for all mutants except amber substitutions. The latter were undetectable on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, except for the amber substitution of the C-terminal Val³¹⁶ codon. Thus, the absence of catalytic activity is not due to a lack of production or stability of the different mutants.

The amino acid replacement sets obtained from mutagenesis of the TS residues studied are shown in Fig. 3. The preliminary results and conclusions are described below. Correlates with x-ray structure refer to the structures of the TS-P_i complex (3) or the *E. coli* TS-dUMP-CB3717 ternary complex (4).

Pro¹⁹⁶ is conserved in 14 of 16 reported TS sequences with Ala in *E. coli* TS and Thr in ϕ 3T TS as known replacements. The side chain of Pro¹⁹⁶ is directed toward the 5'-phosphate of the dUMP-binding site, and the carbonyl oxygen is hydrogen-bonded to a guanidinium nitrogen of the conserved Arg²¹⁸. We obtained 13 mutants, the parental CCG Pro codon, and the amber codon at position 196 after sequencing 29 clones. By complementation, all 13 of the mutants were active, with 12 scoring as highly active and 1 as marginal.

Pro¹⁹⁷ is a completely conserved residue in all reported TS sequences. It resides on β -strand iv, which partially lines the active site. As with Pro¹⁹⁶, the carbonyl oxygen is hydrogen-bonded to Arg²¹⁸ and may play a role in controlling the conformation of the catalytic thiol of the adjacent Cys¹⁹⁸. After sequencing 31 Pro¹⁹⁷ mutants, we identified 13 amino acid substitutions, the amber stop codon, and the CCC Pro codon, which is synonymous with the parental CCG codon. Eight of the mutants were highly active, four scored margin-

Residue	G	A	S	T	C	M	P	N	Q	V	L	I	F	Y	W	D	E	H	R	K
Pro 196	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Pro 197	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Cys 198	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
His 199	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Tyr 146	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Tyr 261	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Asn 229	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Arg 23	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Arg 178	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Arg 179	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Arg 218	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Val 316	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

FIG. 3. Results of complementation screen of mutants in TS-deficient cells. ●, full growth of colonies harboring plasmids; ○, poor growth; ○, no growth. The wild-type mutants isolated are indicated by w within the circles. The Arg¹⁷⁸ mutants were derived from single-strand mutagenesis experiments (8).

ally active, and only one was inactive by complementation. The fact that Pro¹⁹⁷ can be replaced with such a wide variety of residues is surprising since it is buried and is completely conserved in all TS sequences.

Cys¹⁹⁸ provides the catalytic nucleophile of TS and is completely conserved. The catalytic thiol is 3.5–4.5 Å from the guanidinium group of Arg²¹⁸ and could form an ion pair which contributes to reactivity of the thiol. We isolated 11 different substitutions, the synonymous TGC Cys codon, and the amber mutant after sequencing 22 clones. The Ser and Gly mutants were made individually since we failed to readily obtain these mutants from the initial plasmid mixture. None of the Cys¹⁹⁸ mutants could complement *E. coli* Thy⁻ cells, a result which is not surprising considering the crucial role of Cys¹⁹⁸ in catalysis. These observations contrast with those reported for *E. coli* TS (9), where the corresponding Cys-to-Ser mutant complemented a TS deficiency in *E. coli*, and the purified enzyme had ~0.05% of the activity of wild-type TS. We could not detect activity in extracts of the *L. casei* Ser¹⁹⁸ mutant³; perhaps the *E. coli* and *L. casei* enzymes differ in their abilities to accommodate this mutation.

His¹⁹⁹ is conserved in all reported TS sequences, except for the enzyme from bacteriophage ϕ 3T, where the corresponding residue is Val. His¹⁹⁹ lies in the active-site cavity and, from the crystal structures, was considered as the only candidate for a putative general acid/base catalyst to assist the removal of the C-5 proton from dUMP (3). However, the imidazole is too far (5–6 Å) from H-5 of dUMP to act directly in proton transfer, although it could do so through a highly ordered molecule of water bridging to the 4-oxygen of dUMP (4). We isolated 15 mutants of His¹⁹⁹ from some 40 clones sequenced. Five were fully active, and one showed marginal activity in the complementation assay. While this work was in progress, Dev *et al.* (6) reported similar observations at the corresponding His of *E. coli* TS. The six catalytically active mutants we found at position 199 seem to rule out His¹⁹⁹ as an essential general acid/base catalyst, and there are no current alternative candidate residues to serve this function.

Conserved Arg residues at positions 23, 178, 179, and 218 form a positively charged binding surface for the 5'-phosphate of dUMP (3,4). Five of the guanidine NH groups are within appropriate distance (2.5–3.2 Å) to provide hydrogen bonding or electrostatic stabilization of the phosphate anion of dUMP (4). Recently, we reported (8) that Arg¹⁷⁹ can be replaced by amino acids as diverse as Ala and Glu without dramatic effects on binding or catalysis. For Arg¹⁷⁸, after sequencing 30 clones, we isolated 10 mutants, the amber codon, and three synonymous Arg codons. Two mutants were fully active in the complementation assay. After sequencing 11 clones, we obtained four mutants, the amber codon, and two synonymous Arg codons at position 23; none of the mutants were active by the complementation assay. For Arg²¹⁸, we isolated 14 amino acid mutations and the parental Arg codon at position 218 after sequencing 30 clones; sequencing an additional 43 clones provided no additional mutants. None of the mutants were active in the complementation assay. Thus, of the 4 Arg residues involved in phosphate binding, only Arg²³ and Arg²¹⁸ have thus far been shown to be essential.

Tyr¹⁴⁶ is completely conserved and is located near His¹⁹⁹ and Asn²²⁹ in the three-dimensional structure such that it might interact with dUMP. In the initial mutagenesis of Tyr¹⁴⁶ with mixed oligonucleotides, we only obtained small deletion mutations (1–6 base pairs). This probably occurred because the codon for Tyr¹⁴⁶ is located only 2 base pairs from the end

of the mutagenic cassette (7), limiting the ability of the mismatched oligonucleotides to anneal productively. We repeated the experiment using a DNA cassette in which the bottom strand contained inosine at the three positions encoding Tyr¹⁴⁶ and the top strand carried the codon mixture NNG/C (12). Using this approach, we isolated 3 of the 20 possible amino acid mutations, the parental Tyr, and the amber codon; the three mutants were active by the complementation assay.

Tyr²⁶¹ is a completely conserved residue which forms a hydrogen bond to the 3'-hydroxyl of dUMP in the ternary complex. We isolated 14 amino acid mutations, the wild-type Tyr, and the amber codon at position 261 after sequencing 41 clones. Of these, only the Met mutant was active by complementation.

Asn²²⁹ is completely conserved and lies in the highly conserved, hydrophobic J-helix which forms the core of TS. In the ternary *E. coli* TS·dUMP·CB3717 complex, Asn²²⁹ interacts with bound dUMP via hydrogen bonds between the γ -oxygen of Asn and NH-3 of dUMP and between γ -NH₂ and O-4. We isolated 9 of the 20 possible amino acid substitutions, the parental Asn, and the amber codon after sequencing 13 clones. Five of these were active by complementation.

The carboxyl terminus of most TS sequences is Val, although Ile, Leu, and Ala have been found. It appears that the carboxyl-terminal residue is important to TS since removal of Val³¹⁶ in *L. casei* TS by carboxypeptidase leads to a loss of folate cofactor binding and catalytic activity (15). Interestingly, on forming TS·dUMP·CB3717, the carboxyl-terminal residues undergo a large movement toward the active site where they approach the folate analog (4). The terminal Val³¹⁶ carboxylate is hydrogen-bonded to the essential Arg²³ and conserved Trp⁸⁵ which probably stabilize the conformer. We isolated 14 amino acid mutations at position 316, the wild-type Val, and the amber codon after sequencing 29 clones. Of these, nine were active by complementation. As expected, the amber mutant lacking terminal residue 316 was not active by the complementation assay.

SUMMARY

We have described a simple strategy for saturation mutagenesis of any residue of TS and for the rapid identification of mutants which are catalytically active. The targeted codon of a synthetic gene is replaced by a mixture of duplex oligonucleotides which encodes all 20 amino acids. After isolation of individual mutants, a complementation assay using TS-deficient *E. coli* rapidly identifies those mutants with catalytic activity.

The important components of the approach we have used which make it generic include the following. (a) A synthetic gene with conveniently placed, unique restriction sites permits mutagenesis at any position at nearly 100% mutagenesis efficiency. (b) A mutagenesis vector which also serves as a high expression vector avoids further subcloning. (c) A selection system for the desired property permits rapid identification of mutants of interest.

We have made replacement sets of amino acid substitutions at 12 conserved residues that are believed to play roles in structure and function of TS. We isolated some 125 different site-directed mutants, 57 (46%) of which were catalytically active. With the exception of Cys¹⁹⁸, Arg²³, and Arg²¹⁸, none of the residues that were mutagenized are strictly essential for activity. All of the active replacement positions had mutants with at least 1% of the activity of wild-type TS, and many have been found with k_{cat} values approaching that of the natural enzyme.³ Despite the wealth of information available on TS, it would not have been possible to predict the

³ S. Climic, L. Ruiz-Perez, D. Gonzalez-Pacanowska, P. Prapunwattana, S.-W. Cho, R. Stroud, and D. V. Santi, unpublished data.

range of allowable substitutions, and the results stand as a caution to assigning structure-function roles of a given residue based on one or a few mutants. To explain how TS can tolerate modifications at so many conserved residues of presumed importance, we suggest that there is a high plasticity of the structure of this protein in accommodating function; that is, when a mutation is made that is potentially detrimental to function, the structure may be sufficiently flexible to accommodate the change. Furthermore, it appears that many of the amino acids studied may not simply serve a role in catalysis, or else they would not have survived evolutionary pressure. We are searching for other contributing factors to the conservation of these residues.

REFERENCES

1. Santi, D. & Danenberg, P. (1984) in *Chemistry and Biochemistry of Folate* (Blakely, R. & Benkovic, S., eds) pp. 345-398, John Wiley & Sons, New York.
2. Perryman, S., Rossana, C., Deng, T., Vanin, E. & Johnson, L. (1986) *Mol. Biol. Evol.* **3**, 313-321.
3. Hardy, L., Finer-Moore, J., Montfort, W., Jones, M., Santi, D. & Stroud, R. (1987) *Science* **235**, 448-455.
4. Montfort, W., Perry, K., Fauman, E., Finer-Moore, J., Maley, G., Hardy, L., Maley, F. & Stroud, R. (1990) *Protein-Struct. Funct. Genet.*, in press.
5. Matthews, D., Appelt, K. & Oatley, S. (1989) *Adv. Enzyme Regul.* **29**, 47-60.
6. Dev, I. K., Yates, B. B., Atashi, J. & Dallas, W. S. (1989) *J. Biol. Chem.* **264**, 19132-19137.
7. Climie, S. & Santi, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 633-637.
8. Santi, D., Pinter, K., Kealey, J. & Davisson, V. (1990) *J. Biol. Chem.* **265**, 6770-6775.
9. Dev, I., Yates, B., Leong, J. & Dallas, W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1472-1476.
10. Schultz, S. & Richards, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1588-1592.
11. Wells, J., Vasser, M. & Powers, D. (1985) *Gene (Amst.)* **34**, 315-323.
12. Reidhaar-Olson, J. & Sauer, R. (1988) *Science* **241**, 53-57.
13. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1988) *Currents Protocols in Molecular Biology*, John Wiley & Sons, New York.
14. Wells, J., Cunningham, B., Graycar, T. & Estell, D. (1986) *Philos. Trans. R. Soc. Lond. A Math. Phys. Sci.* **317**, 415-423.
15. Aull, J. L., Loeb, R. B. & Dunlap, R. B. (1974) *J. Biol. Chem.* **249**, 1167-1172.
16. Michaels, L. M., Kim, C. W., and Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3957-3961.

STIC-ILL

Mucally

From: Steadman, David (AU1652)
Sent: Wednesday, February 13, 2002 8:09 AM
To: STIC-ILL
Subject: Literature/ILL request

Art Unit: 1652
Office: 10D-04
Mailbox: 10C-01 M3
Case Serial #:09/720,096

Please provide the following reference:

J Biol Chem 1990 Nov 5;265(31):18776-9
Saturation site-directed mutagenesis of thymidylate synthase.
Climie S, Ruiz-Perez L, Gonzalez-Pacanowska D, Prapunwattana P, Cho SW, Stroud R, Santi DV.

Thank you very much,
David J. Steadman
Art Unit 1652
CM1, 10D-04
308-3934

Saturation Site-directed Mutagenesis of Thymidylate Synthase*

(Received for publication, May 18, 1990)

Shane Climie†, Luis Ruiz-Perez‡, Dolores Gonzalez-Pacanowska§, Phisit Prapunwattana‡, Sung-Woo Cho‡, Robert Stroud†, and Daniel V. Santi‡§

From the †Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0448 and the ‡Institute Lopez-Neyra of Parasitology, Consejo Superior de Investigaciones Científicas, Granada, 18001 Spain

We have subjected 12 different codons of a synthetic *Lactobacillus casei* thymidylate synthase (TS) gene to saturation site-directed mutagenesis to create amino acid "replacement sets" at each of those positions. The target residues were chosen because they are highly conserved and because they are important for the structure and function of the protein as indicated by solution and structural studies. The mutagenesis procedure involved excision of a fragment of the synthetic gene containing the target codon, followed by its replacement with a mixture of oligonucleotides which code for all 20 amino acids and the amber stop codon. TS mutants were identified by DNA sequencing, and catalytically active mutants were identified by genetic complementation using a *Thy*⁻ strain of *Escherichia coli*. Only 3 of the 12 target amino acids examined were essential for TS activity; and of the 125 total mutants identified, 57 were catalytically active. These results point to a high degree of plasticity of TS in accommodating function with structural change.

Thymidylate synthase (TS)¹ catalyzes the conversion of 2'-deoxyuridylyl (dUMP) and 5,10-methylenetetrahydrofolate to 2'-deoxythymidylyl (dTMP) and dihydrofolate. TS is the sole *de novo* source of dTMP, and there has been much interest in studies of its structure, function, and inhibition. In part, the interest in TS results from the large amount of information on this enzyme which now exists. The enzymology of TS has been extensively studied, and details about its mechanism and inhibition are well understood (1). Also, the primary sequences of TS from some 16 sources have been determined and have revealed that it is the one of most conserved proteins known (2); the similarity among TS sequences implies a functional importance for the conserved residues. Finally, the x-ray structures of the *Lactobacillus casei* TS-P₁ complex and the *Escherichia coli* TS-dUMP-10-propargyl-5,8-dideazafolate (CB3717) ternary complex have provided insight about the possible roles of specific residues of the protein (3-5).

Thus far, mutations of key residues of TS have provided unexpected results: His¹⁰⁰, a putative general base catalyst (6,

7), and Arg¹⁷⁹, a substrate-binding residue (8), could each be substituted by several different residues without large effects on binding or catalysis; even the Cys involved in nucleophilic catalysis could be modified to Ser and still retain activity, albeit severely diminished (9). From such observations, we became cautious about directing our efforts toward the study of one or a few mutants. Instead, we adopted a plan to produce all possible mutants at a residue of interest and developed screening methods for various properties to reveal mutants of interest.

In this report, we describe the strategy and preliminary results of an approach to understanding the structure of TS by saturation site-directed mutagenesis. The mutations were constructed by cassette mutagenesis using an *E. coli* expression vector that carries a synthetic *L. casei* TS gene with over 30 unique restriction sites (7). The mutagenic DNA cassettes contained mixtures of 32 codons that encode 20 amino acids and the amber (TAG) stop codon at the target site (10-12). "Replacements sets" were constructed in which each of 12 target amino acids were replaced by a large number of substitutions, and a complementation assay was used to rapidly screen for mutants that were catalytically active and thus of more interest for further study.²

MATERIALS AND METHODS

Restriction endonucleases and T4 ligase were purchased from New England Biolabs and Bethesda Research Laboratories and were used, as recommended. The Sequenase DNA sequencing kit was purchased from U. S. Biochemical Corp. The *E. coli* strains used were TB-1 (φ80lacZΔM15; ara, Δ(lac-proAB), rpsL, hsdR) from T. O. Baldwin (Texas A & M, College Station, TX) and *Thy*⁻χ2913 (ΔthyA572) from R. Thompson (University of Glasgow, United Kingdom). Oligonucleotides were synthesized at the University of California-San Francisco Biomolecular Resource Center using an Applied Biosystems 380B DNA synthesizer; tritylated oligonucleotide mixtures were purified and de-tritylated on Du Pont-New England Nuclear NEN-SORB Prep cartridges using the recommendations of the manufacturer. General methods for plasmid purification, subcloning, and bacterial transformation were as described (13).

In vitro cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9 (7). In some cases, pSCTS9 was first modified by substituting a 19-base noncoding sequence containing unique *NotI* and *SphI* sites for the fragment containing the target codon; this eliminates wild-type sequences and allows for selective destruction of the parent plasmid and resultant increase in mutagenesis efficiency (14). Methods for the construction and characterization of the TS mutants were as described (7). Short fragments of the TS gene containing the target codon were replaced with a synthetic DNA duplex presenting a mixture of all four bases at the first and second positions of the target codon and a mixture of G and C at the third position. The plasmids from the ligation mixture were used to trans-

* This work was supported by United States Public Health Service Grant CA14394 and the TDR/Rockefeller Foundation Joint Venture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Senior Fulbright/Spanish Ministry of Education and Science Scholar.

‡ To whom reprint requests should be addressed.

§ The abbreviation used is: TS, thymidylate synthase.

² The use of a series of amber tRNA suppressors and genetic complementation to construct and characterize a large number of mutants of *E. coli* TS was reported after this manuscript was submitted (10).

form *E. coli* TB-1, and plasmid pools were transformed into *E. coli* Thy⁻ χ 2913. Individual plasmids were twice re-transformed into χ 2913 and sequenced. Catalytically active substitutions were identified by screening for the ability of Thy⁻ χ 2913 cells harboring the mutant plasmids to grow on minimal agar lacking Thd and were again characterized by DNA sequencing.

RESULTS AND DISCUSSION

The amino acids targeted for mutagenesis were chosen because they are highly conserved and because they have putative roles in substrate or cofactor binding or in catalysis, as determined by solution or structural studies (Fig. 1). The strategy used in the construction and characterization of the replacement sets is outlined in Fig. 2. Following ligation of oligonucleotide mixtures and initial transformation, colonies were pooled, and the mutagenized plasmid DNA pool was recovered and used to transform *E. coli* Thy⁻ χ 2913 to ampicillin resistance. Individual mutants were identified by DNA sequencing, and the resulting plasmids were used to re-transform χ 2913. Plasmid DNA from the secondary χ 2913 transformants was again sequenced and tested for TS activity as described below. Passage of the mutagenized DNA mixtures through several rounds of transformation ensured segregation and/or repair of the heteroduplex DNA molecules that were created during the construction of the mutants. It was not practical to identify all possible mutants in a replacement set by this strategy, which would require sequencing of over 160 clones to obtain a 95% confidence level of obtaining a complete set. Generally, 20–30 isolates of a replacement set were sequenced to give 10–15 different mutants of a given residue.

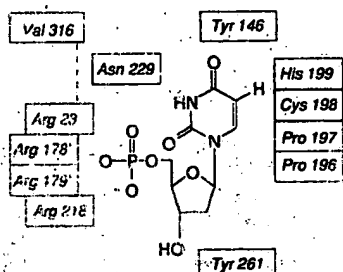


FIG. 1. Amino acid residues of TS targeted for mutagenesis depicted near the sites of interaction with dUMP. ---, hydrogen bond between Val³¹⁶ and Arg²³.

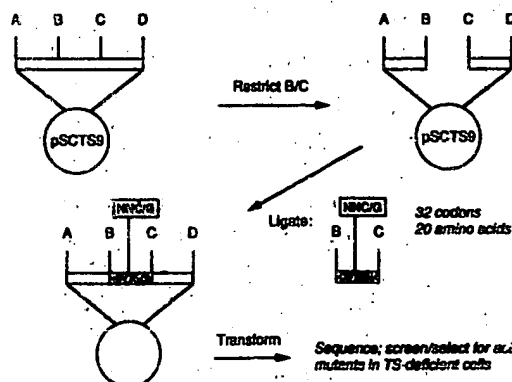


FIG. 2. Strategy for saturation site-directed mutagenesis of synthetic TS gene and identification of catalytically active mutants. A fragment of the TS gene between any two restriction sites is removed and replaced by a mixture of oligonucleotides containing NNC/C at the target codon. The resultant plasmids are sequenced and screened in *E. coli* Thy⁻ cells for catalytic activity.

An important step in our strategy involves the identification of catalytically active mutants by genetic complementation of *E. coli* χ 2913, which is deficient in TS. Individual mutant isolates were grown under nonselective conditions and then patched onto minimal agar lacking Thd. Cell growth in the absence of exogenous Thd identifies mutants that express catalytically active TS. Mutants were scored for full growth, weak growth, or no growth. A comparison of the specific activity (data not shown) of TS in crude extracts from a variety of mutants indicated that a specific activity of $\sim 0.002 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ($0.001\text{--}0.005 \mu\text{mol min}^{-1} \text{mg}^{-1}$) is necessary for complementation of χ 2913; this corresponds to $\sim 1\%$ of the activity of cells harboring the parent plasmid pSCTS9.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell extracts showed that TS was expressed at levels of 5–30% of the total soluble protein for all mutants except amber substitutions. The latter were undetectable on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, except for the amber substitution of the C-terminal Val³¹⁶ codon. Thus, the absence of catalytic activity is not due to a lack of production or stability of the different mutants.

The amino acid replacement sets obtained from mutagenesis of the TS residues studied are shown in Fig. 3. The preliminary results and conclusions are described below. Correlates with x-ray structure refer to the structures of the TS-P_i complex (3) or the *E. coli* TS-dUMP-CB3717 ternary complex (4).

Pro¹⁹⁶ is conserved in 14 of 16 reported TS sequences with Ala in *E. coli* TS and Thr in ϕ 3T TS as known replacement. The side chain of Pro¹⁹⁶ is directed toward the 5'-phosphate of the dUMP-binding site, and the carbonyl oxygen is hydrogen-bonded to a guanidinium nitrogen of the conserved Arg²¹⁸. We obtained 13 mutants, the parental CCG Pro codon, and the amber codon at position 196 after sequencing 29 clones. By complementation, all 13 of the mutants were active, with 12 scoring as highly active and 1 as marginal.

Pro¹⁹⁷ is a completely conserved residue in all reported TS sequences. It resides on β -strand iv, which partially lines the active site. As with Pro¹⁹⁶, the carbonyl oxygen is hydrogen-bonded to Arg²¹⁸ and may play a role in controlling the conformation of the catalytic thiol of the adjacent Cys¹⁹⁸. After sequencing 31 Pro¹⁹⁷ mutants, we identified 13 amino acid substitutions, the amber stop codon, and the CCC Pro codon, which is synonymous with the parental CCG codon. Eight of the mutants were highly active, four scored margin-

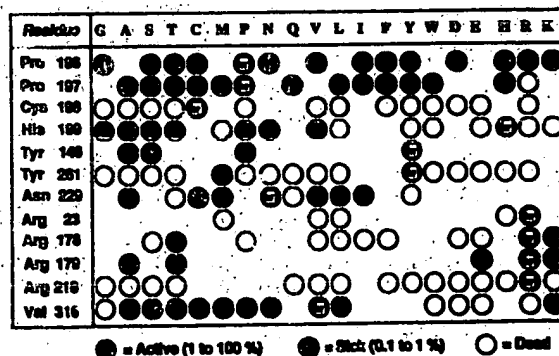


FIG. 3. Results of complementation screen of mutants in TS-deficient cells. ●, full growth of colonies harboring plasmids; ○, poor growth; ○, no growth. The wild-type mutants isolated are indicated by w within the circles. The Arg²¹⁸ mutants were derived from single-strand mutagenesis experiments (8).

ally active, and only one was inactive by complementation. The fact that Pro¹⁹⁷ can be replaced with such a wide variety of residues is surprising since it is buried and is completely conserved in all TS sequences.

Cys¹⁹⁸ provides the catalytic nucleophile of TS and is completely conserved. The catalytic thiol is 3.5–4.5 Å from the guanidinium group of Arg²¹⁸ and could form an ion pair which contributes to reactivity of the thiol. We isolated 11 different substitutions, the synonymous TGC Cys codon, and the amber mutant after sequencing 22 clones. The Ser and Gly mutants were made individually since we failed to readily obtain these mutants from the initial plasmid mixture. None of the Cys¹⁹⁸ mutants could complement *E. coli* Thy⁻ cells, a result which is not surprising considering the crucial role of Cys¹⁹⁸ in catalysis. These observations contrast with those reported for *E. coli* TS (9), where the corresponding Cys-to-Ser mutant complemented a TS deficiency in *E. coli*, and the purified enzyme had ~0.05% of the activity of wild-type TS. We could not detect activity in extracts of the *L. casei* Ser¹⁹⁸ mutant³; perhaps the *E. coli* and *L. casei* enzymes differ in their abilities to accommodate this mutation.

His¹⁹⁹ is conserved in all reported TS sequences, except for the enzyme from bacteriophage ϕ 3T, where the corresponding residue is Val. His¹⁹⁹ lies in the active-site cavity and, from the crystal structures, was considered as the only candidate for a putative general acid/base catalyst to assist the removal of the C-5 proton from dUMP (3). However, the imidazole is too far (5–6 Å) from H-5 of dUMP to act directly in proton transfer, although it could do so through a highly ordered molecule of water bridging to the 4-oxygen of dUMP (4). We isolated 15 mutants of His¹⁹⁹ from some 40 clones sequenced. Five were fully active, and one showed marginal activity in the complementation assay. While this work was in progress, Dev *et al.* (6) reported similar observations at the corresponding His of *E. coli* TS. The six catalytically active mutants we found at position 199 seem to rule out His¹⁹⁹ as an essential general acid/base catalyst, and there are no current alternative candidate residues to serve this function.

Conserved Arg residues at positions 23, 178, 179, and 218 form a positively charged binding surface for the 5'-phosphate of dUMP (3, 4). Five of the guanidine NH groups are within appropriate distance (2.5–3.2 Å) to provide hydrogen bonding or electrostatic stabilization of the phosphate anion of dUMP (4). Recently, we reported (8) that Arg¹⁷⁹ can be replaced by amino acids as diverse as Ala and Glu without dramatic effects on binding or catalysis. For Arg¹⁷⁹, after sequencing 30 clones, we isolated 10 mutants, the amber codon, and three synonymous Arg codons. Two mutants were fully active in the complementation assay. After sequencing 11 clones, we obtained four mutants, the amber codon, and two synonymous Arg codons at position 23; none of the mutants were active by the complementation assay. For Arg²¹⁸, we isolated 14 amino acid mutations and the parental Arg codon at position 218 after sequencing 30 clones; sequencing an additional 43 clones provided no additional mutants. None of the mutants were active in the complementation assay. Thus, of the 4 Arg residues involved in phosphate binding, only Arg²³ and Arg²¹⁸ have thus far been shown to be essential.

Tyr¹⁴⁸ is completely conserved and is located near His¹⁹⁹ and Asn²²⁹ in the three-dimensional structure such that it might interact with dUMP. In the initial mutagenesis of Tyr¹⁴⁸ with mixed oligonucleotides, we only obtained small deletion mutations (1–6 base pairs). This probably occurred because the codon for Tyr¹⁴⁸ is located only 2 base pairs from the end

of the mutagenic cassette (7), limiting the ability of the mismatched oligonucleotides to anneal productively. We repeated the experiment using a DNA cassette in which the bottom strand contained inosine at the three positions encoding Tyr¹⁴⁸ and the top strand carried the codon mixture NNG/C (12). Using this approach, we isolated 3 of the 20 possible amino acid mutations, the parental Tyr, and the amber codon; the three mutants were active by the complementation assay.

Tyr²⁶¹ is a completely conserved residue which forms a hydrogen bond to the 3'-hydroxyl of dUMP in the ternary complex. We isolated 14 amino acid mutations, the wild-type Tyr, and the amber codon at position 261 after sequencing 41 clones. Of these, only the Met mutant was active by complementation.

Asn²²⁹ is completely conserved and lies in the highly conserved, hydrophobic J-helix which forms the core of TS. In the ternary *E. coli* TS·dUMP·CB3717 complex, Asn²²⁹ interacts with bound dUMP via hydrogen bonds between the γ -oxygen of Asn and NH-3 of dUMP and between γ -NH₂ and O-4. We isolated 9 of the 20 possible amino acid substitutions, the parental Asn, and the amber codon after sequencing 13 clones. Five of these were active by complementation.

The carboxyl terminus of most TS sequences is Val, although Ile, Leu, and Ala have been found. It appears that the carboxyl-terminal residue is important to TS since removal of Val³¹⁶ in *L. casei* TS by carboxypeptidase leads to a loss of folate cofactor binding and catalytic activity (15). Interestingly, on forming TS·dUMP·CB3717, the carboxyl-terminal residues undergo a large movement toward the active site where they approach the folate analog (4). The terminal Val³¹⁶ carboxylate is hydrogen-bonded to the essential Arg²³ and conserved Trp⁶⁵ which probably stabilize the conformer. We isolated 14 amino acid mutations at position 316, the wild-type Val, and the amber codon after sequencing 29 clones. Of these, nine were active by complementation. As expected, the amber mutant lacking terminal residue 316 was not active by the complementation assay.

SUMMARY

We have described a simple strategy for saturation mutagenesis of any residue of TS and for the rapid identification of mutants which are catalytically active. The targeted codon of a synthetic gene is replaced by a mixture of duplex oligonucleotides which encodes all 20 amino acids. After isolation of individual mutants, a complementation assay using TS-deficient *E. coli* rapidly identifies those mutants with catalytic activity.

The important components of the approach we have used which make it generic include the following. (a) A synthetic gene with conveniently placed, unique restriction sites permits mutagenesis at any position at nearly 100% mutagenesis efficiency. (b) A mutagenesis vector which also serves as a high expression vector avoids further subcloning. (c) A selection system for the desired property permits rapid identification of mutants of interest.

We have made replacement sets of amino acid substitutions at 12 conserved residues that are believed to play roles in structure and function of TS. We isolated some 125 different site-directed mutants, 57 (46%) of which were catalytically active. With the exception of Cys¹⁹⁸, Arg²³, and Arg²¹⁸, none of the residues that were mutagenized are strictly essential for activity. All of the active replacement positions had mutants with at least 1% of the activity of wild-type TS, and many have been found with k_{cat} values approaching that of the natural enzyme.³ Despite the wealth of information available on TS, it would not have been possible to predict the

³ S. Climis, I. Ruiz-Perez, D. Gonzalez-Pacanowska, P. Prapunwattana, S.-W. Cho, R. Stroud, and D. V. Santi, unpublished data.

range of allowable substitutions, and the results stand as a caution to assigning structure-function roles of a given residue based on one or a few mutants. To explain how TS can tolerate modifications at so many conserved residues of presumed importance, we suggest that there is a high plasticity of the structure of this protein in accommodating function; that is, when a mutation is made that is potentially detrimental to function, the structure may be sufficiently flexible to accommodate the change. Furthermore, it appears that many of the amino acids studied may not simply serve a role in catalysis, or else they would not have survived evolutionary pressure. We are searching for other contributing factors to the conservation of these residues.

REFERENCES

1. Santi, D. & Danenberg, P. (1984) in *Chemistry and Biochemistry of Folate* (Blakely, R. & Benkovic, S., eds) pp. 345-398, John Wiley & Sons, New York
2. Perryman, S., Rossana, C., Deng, T., Vanin, E. & Johnson, L. (1986) *Mol. Biol. Evol.* **3**, 313-321
3. Hardy, L., Finer-Moore, J., Montfort, W., Jones, M., Santi, D. & Stroud, R. (1987) *Science* **235**, 448-455
4. Montfort, W., Perry, K., Fauman, E., Finer-Moore, J., Maley, G., Hardy, L., Maley, F. & Stroud, R. (1990) *Protein-Struct. Funct. Genet.*, in press
5. Matthews, D., Appelt, K. & Oatley, S. (1989) *Adv. Enzyme Regul.* **29**, 47-80
6. Dev, I. K., Yates, B. B., Atsahi, J. & Dallas, W. S. (1989) *J. Biol. Chem.* **264**, 19132-19137
7. Climis, S. & Santi, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 633-637
8. Santi, D., Pinter, K., Kealey, J. & Davisson, V. (1990) *J. Biol. Chem.* **265**, 6770-6775
9. Dev, I., Yates, B., Leong, J. & Dallas, W. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1472-1476
10. Schultz, S. & Richards, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1588-1592
11. Wells, J., Vassar, M. & Powers, D. (1985) *Gene (Amst.)* **34**, 315-323
12. Reidhaar-Olson, J. & Sauer, R. (1988) *Science* **241**, 53-57
13. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1989) *Currents Protocols in Molecular Biology*, John Wiley & Sons, New York
14. Wells, J., Cunningham, B., Graycar, T. & Estell, D. (1986) *Philos. Trans. R. Soc. Lond. A Math. Phys. Sci.* **317**, 415-423
15. Aull, J. L., Loebis, R. B. & Dunlap, R. B. (1974) *J. Biol. Chem.* **249**, 1167-1172
16. Michaels, L. M., Kim, C. W., and Miller, J. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3957-3961